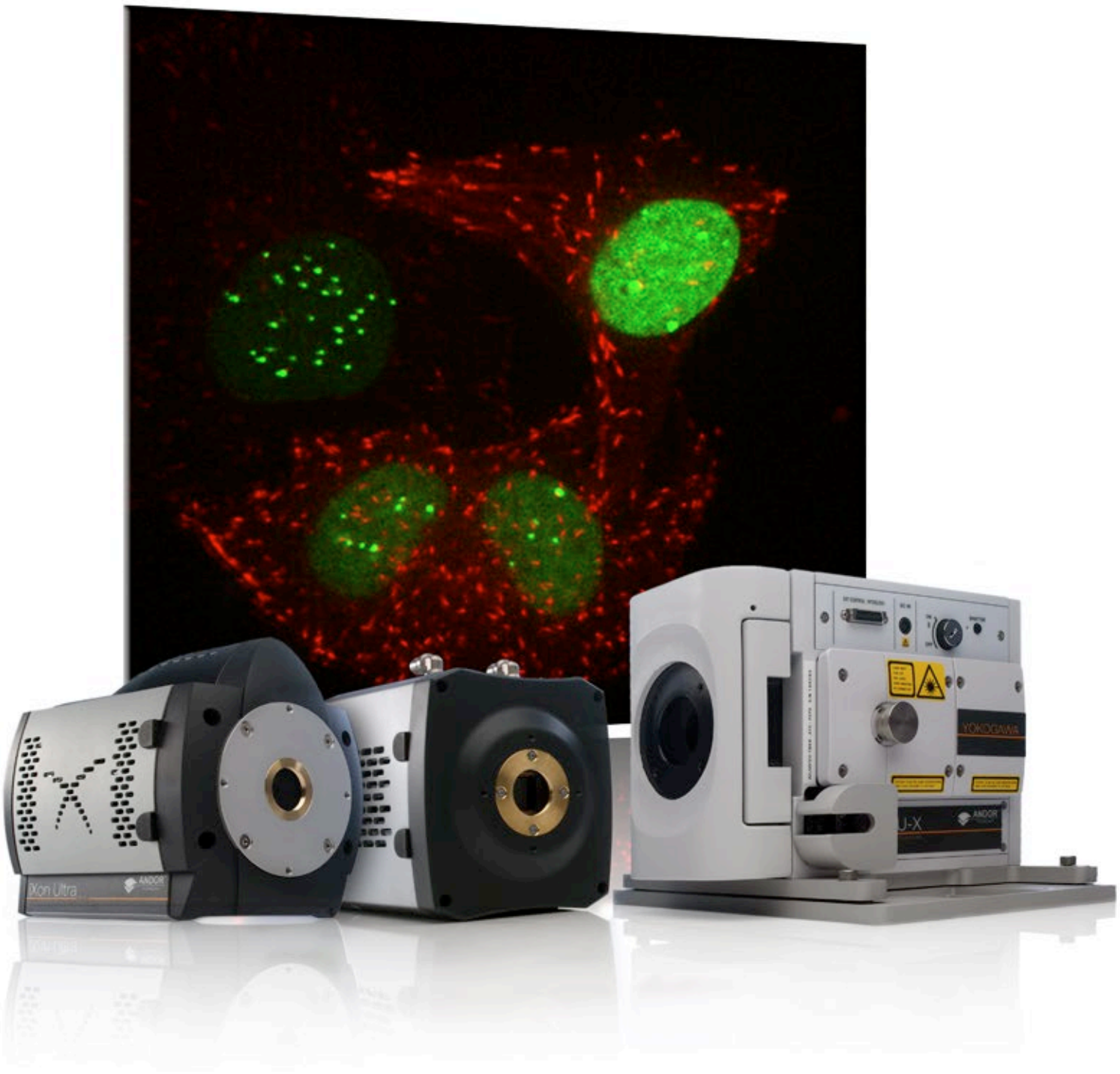
ANDOR™
TECHNOLOGY

Revolution XD

Confocal Spinning Disk Solutions

Setting the standard in high speed imaging



The Revolution XD Family

The only solution when life is so precious

The Andor Revolution XD is a family of flexible system solutions focused on live cell imaging. At the heart of our systems is the CSU-X spinning disk technology from Yokogawa®, the industry leader in this technology, and broadly considered the best choice for sensitive live-cell confocal imaging.

In offering the new Neo sCMOS, iXon Ultra EMCCD and the 10,000 rpm CSU-X, Andor can offer unprecedented image capture rates for your high speed applications such as ion imaging and tracking Manufacturing many of the core system components we can ensure optimum performance and reliability.

Meet the family

Experience tells us that almost every customer is unique in their requirements, and so rarely are two Revolution XD systems the same. We pride ourselves in offering flexibility in the hardware solutions we offer, so that you can focus on your applications. In recognition of this we are proud to introduce the Revolution XD family.

Revolution XDi - The classic configuration on an inverted microscope.
Revolution XDh - Combine full system choice with an upright microscope.
Revolution XDv - A focused upright solution when lab space is at a premium.

Features	Benefits
Yokogawa CSU-X1 spinning disk confocal unit	Ultimate in high speed and low phototoxicity for live cell imaging
iXon EMCCD's with highest sensitivity and speeds	Detect weak signals, minimise bleaching and toxicity, maximise temporal resolution
sCMOS cameras	Nyquist sampling with sensitivity for high resolution confocal imaging
TuCam dual camera adapter	Full frame dual wavelength capture and anisotropy studies (e.g. homo-FRET)
Andor 6-line Laser Combiner with active blanking	Cover a broad range of fluorophores. Reduced photobleaching and toxicity
Andor Multi-Port Unit	Utilise imaging wavelengths for FRAPPA and TIRF devices. Application flexibility minimizing laser costs
Andor filter wheel	Reduce back-reflections for optimum image quality
Photo-stimulation tools for bleaching, activating, switching, ablating, perturbing, uncaging	Reveal the detail behind cellular dynamics across a broad range of models and applications
Andor Precision Control Unit	Fast multidimensional imaging, and additional peripheral device communication and control
Configuration flexibility	Application focused configurations
Open hardware architecture	Easy to upgrade in the future and so grow with your research requirements
Broad software compatibility	Offering you a choice to run your Revolution XD on your preferred software platform

Capturing new detail

Running at 56 frames per second at full frame, the new Andor iXon Ultra offers the fastest speeds available from the most popular detector for spinning disk confocal microscopy. At these higher speeds, you can discover previously unseen events.

Andor's sCMOS technology provides the optimum resolution you need for Confocal imaging. Complimentary to EMCCD technology, sCMOS offers high resolution with excellent signal to noise for spinning disk confocal. Reveal the fine detail you may have been missing.

Revealing hidden processes

Our Active Illumination (AI) portfolio provides tools for research into areas such as damage and repair, cell signaling, cell motility, cellular compartmentalisation, protein dynamics, developmental biology and optogenetics. The techniques employed being photo-bleaching, activation and conversion, photoablation, uncaging and light induced protein control such as receptor activation, protein recruitment, and ion channel control. These tools will give you detailed spatial and temporal insight beyond what is capable with routine imaging.

Focusing on applications, understanding needs

Whether your field of interest is in cancer research, damage and repair, developmental biology, plant research or neuroscience, our focus is to provide you with the tools you need to unravel the cellular processes at the heart of your research.

We understand the fundamental requirements of imaging live samples in a way that preserves their physiology, whilst at the same time unlocking the detail you need to observe, and so uncover, the answers you are looking for.

Revolution XDi

Classic Live Cell Confocal System

The Revolution XDi is based around an inverted microscope, the most common choice for live cell imaging, ideal for samples such as cultured cells that grow in media and require careful environmental control. Samples can range from something as small as yeast through to a large specimen such as drosophila embryo.

Flexibility

The Revolution XD is more than just a spinning disk solution, For membrane related biology we can integrate with TIRF illuminators. Active Illumination devices offer a deeper understanding of cell dynamics and signaling Spinning disk technology does not suit all techniques and so you can run complimentary widefield configurations on the same microscope for applications such as ratiometric calcium imaging. Our application engineers will be happy to consult you and offer advice.

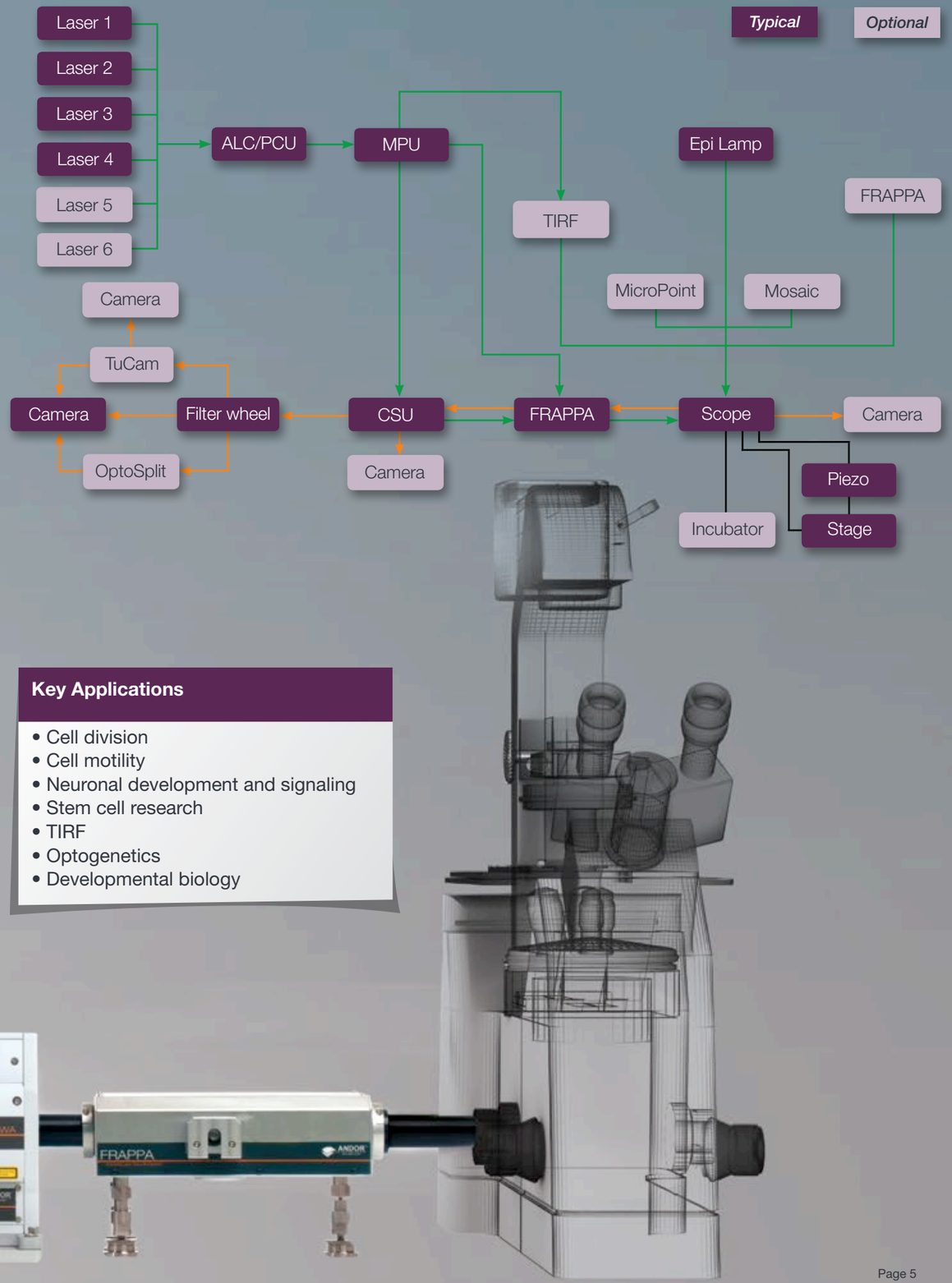
Application focused

With a simply arranged optical path offering optimum light efficiency, we can easily add additional application focused tools into the system. For example, live colocalisation studies such as FRET ideally require two color simultaneous image capture. The Andor TuCam means you can run two cameras simultaneously and capture at full field of view. Active illumination devices such as FRAPPA, Mosaic and MicroPoint are tools that can be added according to your immediate application needs or as your research develops.

> One of the major benefits of the Revolution XD applied to 4D microscopy has to do with the quickness of image acquisition at low intensity illumination. Conventional point scanning confocal microscopy would not have allowed for 4D image acquisition of POLO-depleted mitotic cells without severely impairing cell viability.
>
> Dr Tatiana Moutinho dos Santos PhD, MSc,
> Molecular Genetics Lab IBMC-INEB,
> Porto, Portugal

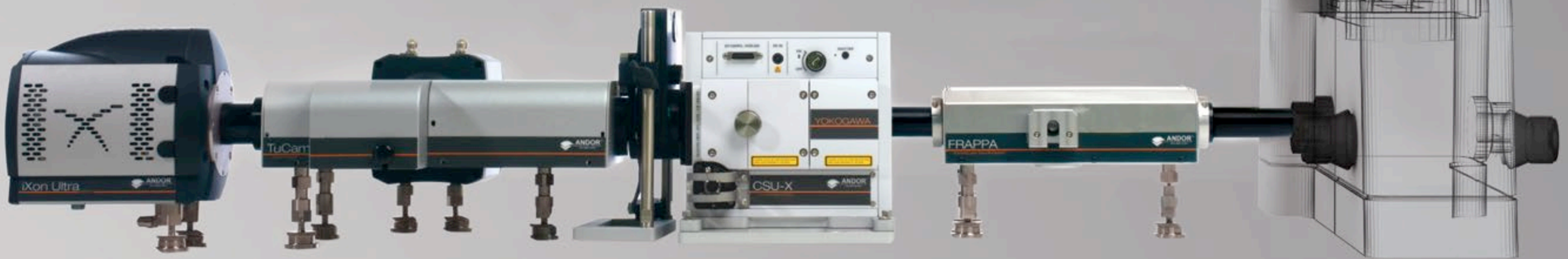
For “Spinning disk confocal microscopy” Application Note click here

XDi System Configurator



Key Applications

- Cell division
- Cell motility
- Neuronal development and signaling
- Stem cell research
- TIRF
- Optogenetics
- Developmental biology



Revolution XDh

Full Featured Upright Microscope System

XDh has been designed to allow those who work on an upright microscope benefit from the capabilities available from our full component range. The XDh is suitable for applications such as plant biology research, and in developmental biology models such as c.elegans and drosophila. In these cases, whilst an inverted microscope configuration is possible, an upright microscope can be more practical owing to the sample preparation with a glass coverslip more typically on the upper facing surface. Other examples of applications requiring an upright microscope configuration can be found on page 8.

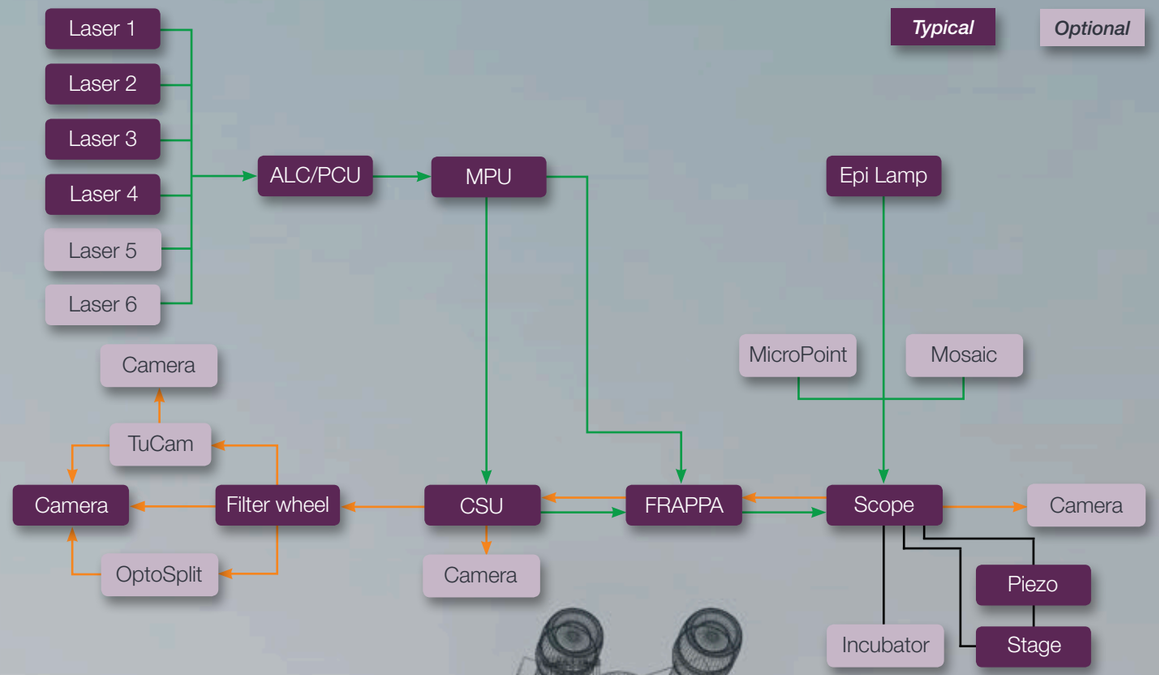
Key Application Example - Plant Biology

Though plants are outwardly slow in their dynamic behaviour, internally they have physiological processes operating at deceptively high speeds, such as can be found in their internal transport systems and at the leading edge of root tips.

In order to investigate such mechanisms of development and homeostasis, you are likely to benefit from the use of devices such as FRAPPA for photo-bleaching and conversion experiments. The Andor FRAPPA device extends the optical train of the Revolution spinning disk system beyond a point that is practical to maintain a vertical arrangement. The XDh solves this limitation.

For movie of Plant Biology sample click here

XDh System Configurator



Key Applications

- Plant research
- Neurophysiology
- Electrophysiology
- Developmental biology
- Intravital tissue imaging
- Vascular physiology

Revolution XDv

Upright Scope With Reduced System Footprint

The Revolution XDv offers you a compact solution on an upright microscope. In keeping this system footprint small, it is well suited to labs with limited space or to fit inside a faraday cage for electrophysiology rigs. In securing the CSU-X, filter wheel and camera to our vertical mount, you are guaranteed complete stability.

Key Application Example - Electrophysiology

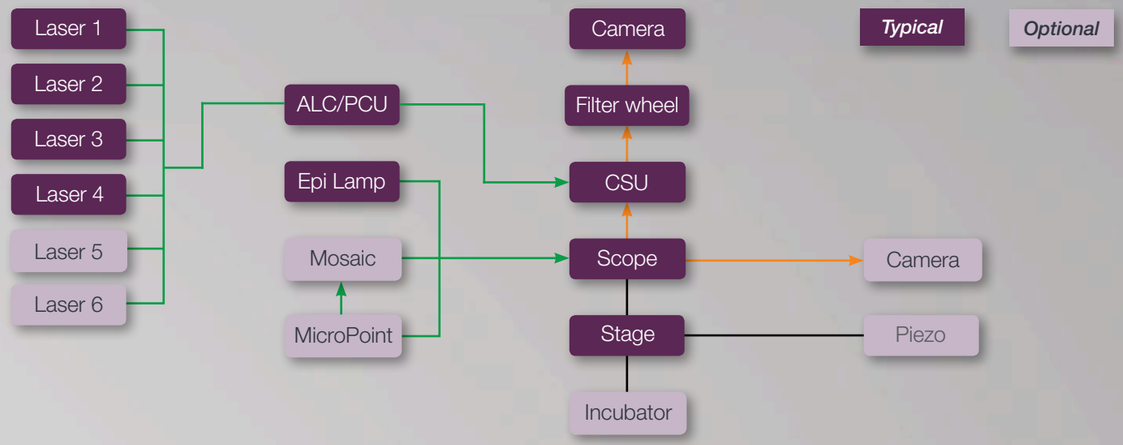
With the Revolution XDv you can confidently combine spinning disk confocal microscopy with techniques as sensitive as patch clamping a cell. Spinning disk technology provides the speed of image capture typically required in association with electrophysiological techniques. Calcium imaging is a popular choice.

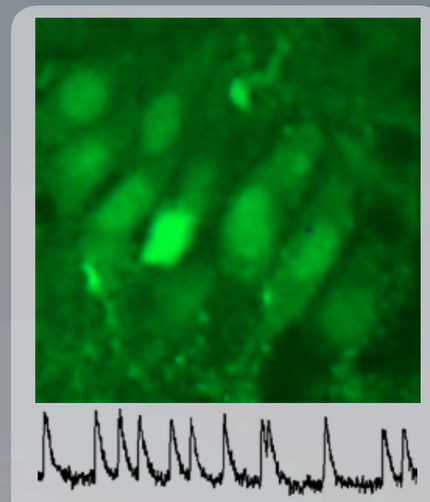
Andor manufactures cameras ideally suited to capture events such as calcium waves and sparks. Often, sample preparations for electrophysiology are too thick for high contrast imaging with conventional fluorescence microscopy, and laser scanning confocal microscopes are too slow for high speed calcium events. Spinning disk technology bridges this gap.

Key Application Example - Intra-vital imaging

For larger systems biology that utilises intra-vital imaging, again the XDv is a well matched configuration. Intra-vital models are used to investigate larger cell populations; structures and organs, such as blood vessels and neurons; and processes such as damage and repair, and immune responses to vessel lesions. With its vertically mounted CSU-X, the XDv keeps the space around the objectives free for the large stage required to take a small mammal and any supporting apparatus.

XDv System Configurator





Hippocampal CA3 neurons loaded with calcium-sensitive OGB-1-AM dye. The activity of a cell was simultaneously imaged (black traces, relative change in intensity) and recorded with an attached recording pipette (red traces, pico amps). Spontaneous action potential firing can be reliably detected from calcium imaging. Imaging was performed at 100 Hz frame rate.

With thanks to János Szabadics and Tibor Andrási of Nikon Microscopy Center at Institute of Experimental Medicine, Budapest.

Custom Systems

At Andor we realize that, sometimes, even our adaptable and flexible off the shelf products are not enough to meet some of the more demanding application requirements of our customers.

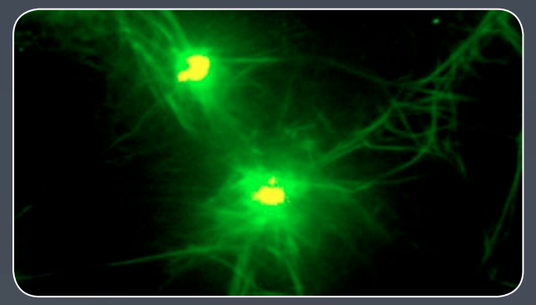
For this reason we provide a bespoke service to our customers, whereby a dedicated highly experienced team of engineers and application specialists provide customer specific solutions. The process involves discussing your core requirements, advising on possible solutions, design development, quotation and final delivery of the product.

We can help you configure custom solutions from any of the components across our entire product range including custom mechanical and optical design.

Our flexible approach and powerful software allows integration of your existing hardware within Andor systems for cost efficiency.

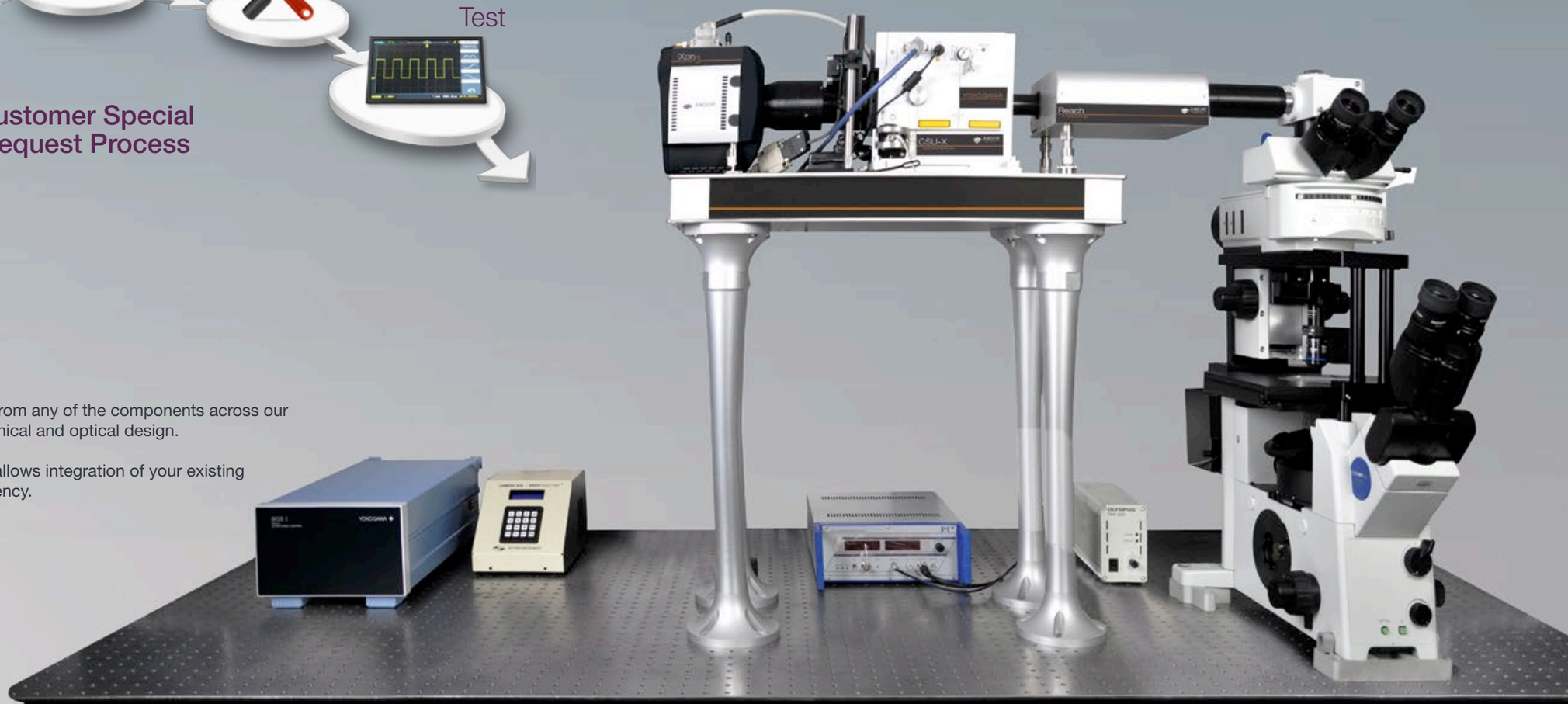
> The flexibility for me to define a novel configuration for the Revolution XD allowed me to combine live cell imaging with laser tweezers. This offers new avenues of investigation for my research.

Dr. Yael Roichman,
School of Chemistry,
Tel Aviv University, Israel

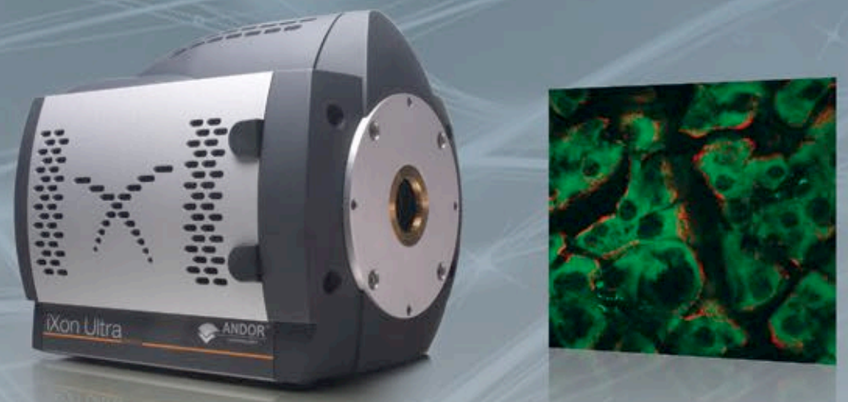


In-vitro actin- myosin-facin network. Actin in green and myosin II in red. Yellow denotes where myosin II complexes are wrapped in actin at network nodes. Image created as part of time series with 100 z-sections captured with a 40x objective.

Example of a custom confocal microscope configuration with upright / inverted Olympus microscope, which was used to implement laser tweezers with SD confocal.



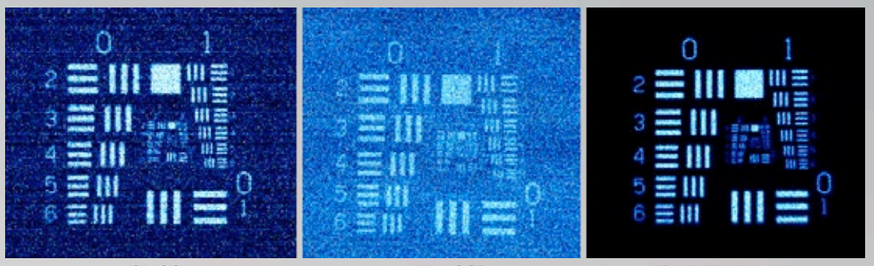
Microscopy System Cameras



iXon EMCCD

EMCCD technology revolutionized spinning disk confocal microscopy. The ability to cool to -100°C and the use of EM gain effectively eliminates noise and provides routine single photon sensitivity. The key benefits of Andor's iXon EMCCD cameras are:

- Extreme sensitivity for working at low levels of sample illumination, hence minimizing phototoxicity; or for detecting weak signals from low expressing fluorescence proteins.
- Imaging at incredibly fast speeds, even at low light levels, in applications such as Calcium Imaging.

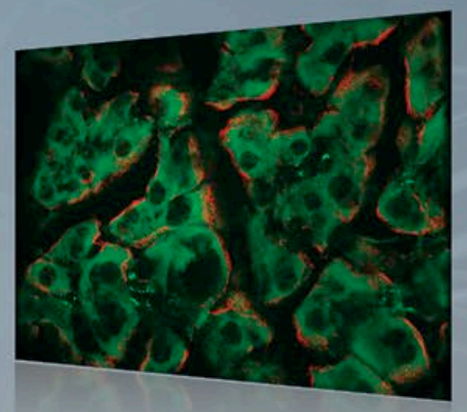


sCMOS (1.2 e^- noise) | Interline CCD (5 e^- noise) | Back illuminated (<1 e^- noise)

Equivilent illumination conditions

For "Comparing sCMOS with other scientific detectors" Technical Note click here

In addition to the iXon Ultra and Neo sCMOS, we also have a range of EMCCD, sCMOS and Interline Cameras.



Neo sCMOS

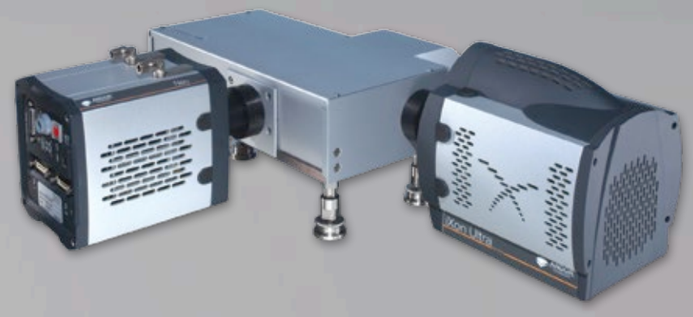
The Neo sCMOS camera is the perfect partner to the iXon range. Though not as sensitive as EMCCD cameras, sCMOS runs at high speeds and has extremely low read noise at 1 e^-/pix/sec, giving excellent signal to noise performance. The key benefits of Neo sCMOS are:

- Small pixels (6.5 µm) capable of Nyquist sampling therefore maximizing on confocal resolution for stunning detailed images.
- Large sensor for increased field of view both on spinning disk and especially standard imaging ports.

Get the best of both worlds

The Andor TuCam dual camera adapter is ideal for large sensors and can be used for more than one purpose.

- Easily switch between cameras with different pixel size and attributes.
- Using two identical cameras simultaneously image two different wavelengths (e.g.co-localization studies) or polarization states (e.g. anisotropy).



Software

Flexibility in your choice of software as well as hardware.

Instrument Control and Data Acquisition

Andor iQ

iQ's flexible protocol architecture can be easily tailored to your specific experiment, including ratio-metric imaging, FRAP analysis and other complex setups. Its unique image disk can handle massive 6D data sets.

iQ can utilise Python scripting, offering links to other packages, and can seamlessly integrate with Imaris for rendering and complex 3D analysis.

MetaMorph

Molecular Devices' MetaMorph software is a popular and familiar interface for many users. Metamorph supports a broad range of third party components with the Revolution XD and Active Illumination portfolio supported.

You can benefit from having a Revolution XD system running in a software environment you are familiar with, as well as all the additional benefits it offers.

MicroManager

MicroManager is a popular open-source software solution within the research community. MicroManager is used for integrating hardware control with image capture, so offering a "homebuild" solution to meet your own specific needs.

MicroManager has integrated core Revolution XD components, and we continue to work with the team to add to the list of supported devices.

NIS Elements

Many users have a preference for Nikon's microscopes and NIS Elements software. When using NIS Elements you will have access to all Nikon components, and can include most of the core components of the Revolution XD.

NIS Elements offers a comprehensive and simple GUI for a range of multi-dimensional imaging tasks, processing and measurements.

LabVIEW

LabVIEW can often provide a suitable development environment for researchers who want to create custom software for their preferred hardware.

Its graphical development environment, integration, with a wide range of hardware along with extensive signal processing and analysis functionality, can make LabVIEW an attractive option. Drivers are available for most Revolution XD components.

Third Party Software Compatibility

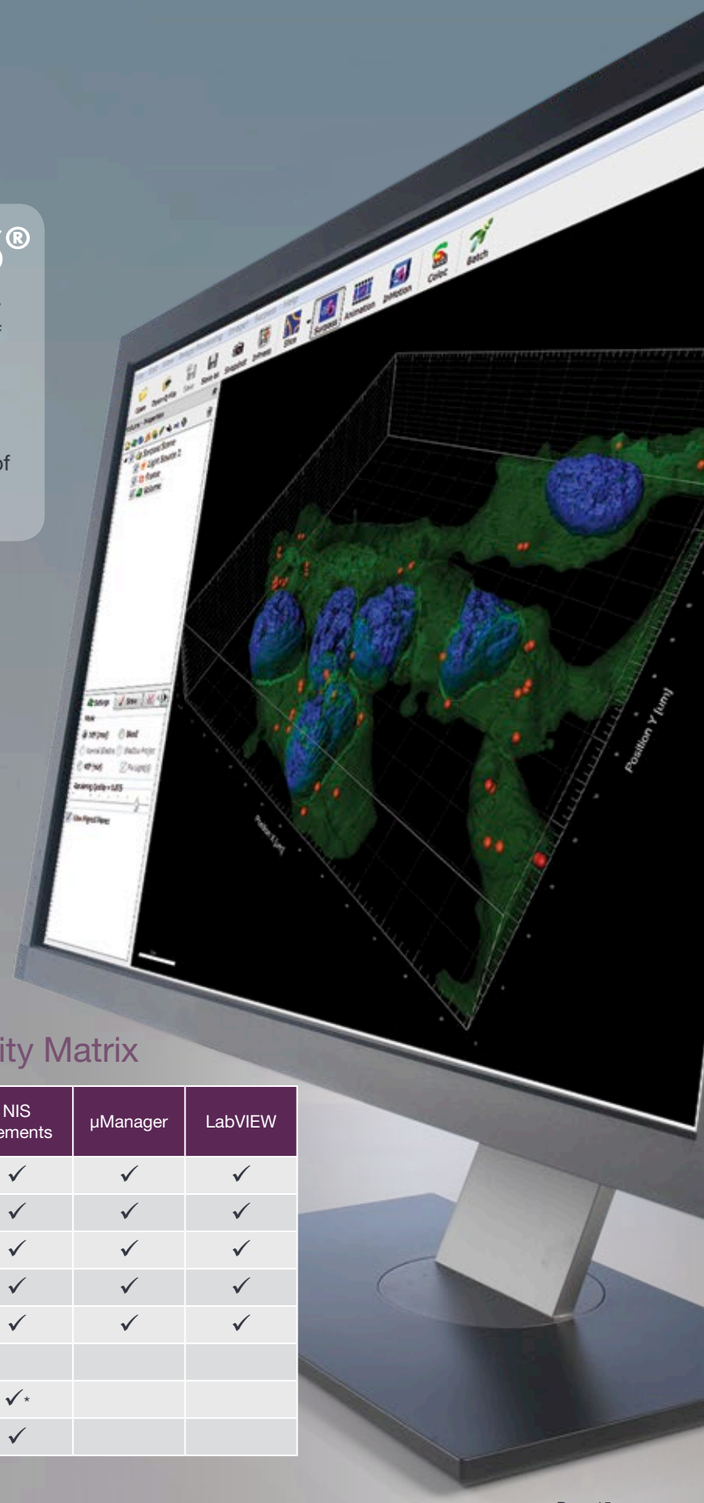
The range of third party software for running Andor products are expanding steadily. Please enquire for further details.

Data Visualization and Analysis

Bitplane Imaris IMARIS®

Imaris delivers all the necessary functionality for visualization, segmentation and interpretation of multidimensional datasets.

By combining speed, precision and intuitive ease-of-use, Imaris provides a complete set of features for handling multi-channel image sets of any size up to 50 gigabytes.



Software / Hardware Compatibility Matrix

	iQ2	Metamorph 7.x	Metamorph NX	NIS Elements	µManager	LabVIEW
iXon3	✓	✓	✓	✓	✓	✓
iXon Ultra	✓	✓	✓	✓	✓	✓
Neo sCMOS	✓	✓	✓	✓	✓	✓
CSU-X1	✓	✓	✓	✓	✓	✓
ALC / PCU	✓	✓	✓	✓	✓	✓
FRAPPA	✓	✓				
MicroPoint	✓	✓		✓*		
Mosaic	✓	✓		✓		

* Only compatible up to version 3 build 728

Microscopy System Components

CSU-X1

Confocal Spinning Disk Unit

The CSU-X1 from Yokogawa is the premier solution for high speed - low photo toxicity imaging. Available in a wide range of configurations, Manual and Motorised versions that can facilitate Confocal Fluorescence imaging as well as Widefield bypass, DIC and SpiDA Anisotropy add-on, which is exclusive to Andor, see Technical Note on page 28.

When performing simultaneous multi-color experiments we recommend use of the TuCam dual cameras adapter, if a third camera is required for three color experiments the third camera can be directly mounted to the CSU. See the TuCam section on page 19.

Features

- 1,500 - 10,000 rpm disk, permitting image acquisition up to 2,000 fps
- Easy synchronization of exposure to disk speed
- Optional bright field bypass module
- FWHM Z section confacility of <= 1.2 µm
- Optimised, Andor specific, dichroic and emission filter pairs.
- The only CSU with Anisotropy

Filter Wheels

Emission discrimination

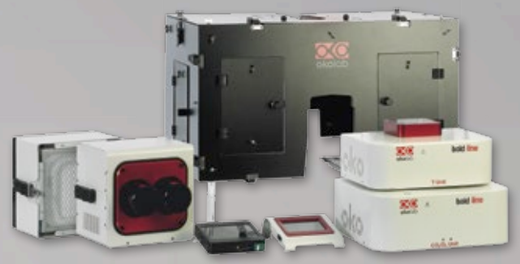
The Revolution XD is available with a broad range of fast filter wheels. Mounted at an angle between the CSU and camera, our design provides optimum filter performance with no back reflections.

Features

- 6,10 and 12 position, o25 filter wheels
- Accommodates emission and DIC filters
- Infinity optical path for optimum filter performance

Stage Incubator

The CO_2 Microscope Stage Incubator (MSI) is a very compact solution to create a suitable environment for cell cultures right on the microscope stage, allowing cells to proliferate as well as they do in a regular bench-top incubator. Humidifying and preheating options prevent medium evaporation and avoid condensation.



Features

- Available for Piezo inserts
- Electric, Water, and Cryo options
- Options include heating and cooling between 10 to 50 °C and regulation down to ±0.1°C
- CO_2 range adjustable between 0% and 100%

Mosaic

Active Illumination

The Mosaic active illumination system utilizes digital mirror device (DMD) technology to control the illumination field of a fluorescence microscope. Using laser or arc lamp sources, Mosaic achieves real time and near diffraction limited resolution. Unlike traditional galvo-scanning systems where pixels are addressed sequentially, Mosaic can simultaneously and precisely excite multiple regions of interest with complex geometries (parallel multi-region illumination) and allow simultaneous imaging. Mosaic is unique, yet flexible, operating over wavelengths ranging from 380 - 800 nm (365 nm on special request).

Features

- Unlimited flexibility in shape and complexity of illumination mask
- No scanning - Simultaneous illumination of multiple regions of interest
- Applications include channelrhodopsin, glutamate uncaging, photoactivation
- Ideal for uncaging, photo-conversion, activation and bleaching

MicroPoint

Laser Illumination and Ablation

MicroPoint is a pulsed laser delivery, which utilises a pulsed N_2 laser to pump a dye cell resonator, yielding pulsed laser output at more than 20 user-exchangeable wavelengths ranging from 365 to 656 nm.

MicroPoint's variable wavelengths and diffraction limited output makes it an excellent photo-stimulation tool providing ablation, uncaging, activation and bleaching capabilities.

Features

- Simultaneous laser delivery and image acquisition
- Ablation, uncaging, activation and bleaching
- 365 - 656 nm - adapt to specific targets
- Incremental control of pulse energy

FRAPPA

Photo-Bleach and Activate

Andor's FRAPPA uses a dual galvanometer scan head to provide a computer-steered laser beam delivery system. By utilising the ALC's range of lasers the FRAPPA provides unrivalled Fluorescence Recovery After Photo-bleach (FRAP) and Photo Activation (PA) flexibility.

The FRAPPA provides diffraction limited performance and can be configured in-line with a CSU or on another ports.

Features

- All ALC laser lines available for FRAP and PA actions
- <10 ms switch over from Imaging to FRAP
- Arbitrary multi-region scanning of points, rectangles and polygons
- Integration with flexible protocols for 3D FRAPPA localization and analysis

Microscopy System Components

Laser Combiner and Multi-Port Unit

Compact, flexible, robust

The ALC can accommodate up to six solid state lasers from 405 – 640 nm, and the Multi port unit facilitates fast switching between up to three channels permitting the laser combiner unit to be used for confocal imaging, FRAP/PA and TIRF.

Laser power control, modulation and microsecond switching are performed using an integral AOTF.

Features

- Hardware blanking minimizes specimen exposure
- Long life solid state lasers with excellent stability (typical ± 0.5% peak to peak)
- Up to six solid state lasers (from 405, 445, 488, 491, 515, 532, 561, 594, 640 nm)
- Powers from 50 - 250 mW depending on wavelength
- Compact 19” rack mount enclosure

Precision Controller Unit

Flexible Expansion

The PCU is a versatile control unit used for the triggering of the Revolution and third party devices.

Features

- Provides complete system synchronization
- Optional 16 bit analogue output for direct Piezo Z control
- Eight digital inputs and eight digital outputs for sensing and triggering external events
- Enables Revolution to slave or master

Motorized XY and Z Control

Automation

We offer a motorized X, Y and Z control applicable to inverted and upright microscope solutions.

XY Stage

Features

- Open and closed loop stages
- Travel >100 x 75 mm @ 30 mm / sec
- 0.02 µm resolution, repeatability 0.2 - 0.3 µm rms
- Perform multi-field scans for 6D imaging
- Create 4D mosaics using iQ software

Z Stage

Features

- 100, 200 and 500 µm ranges
- 1 nm repeatability
- 2 - 8 nm resolution (depends on range)
- Up to 100 Z sections / s
- Accommodates slides, petri and microtitre plates

Piezo Objective

Features

- 100 or 400 µm travel
- Up to 20 Z sections / s
- 1.25 nm resolution
- Analogue or digital control
- Oil and water objectives

Light Sources

We offer a broad portfolio of illumination sources from LED to lasers and Xenon or Metal Halide lamp solutions. This range provides speed and versatility for experiments requiring high brightness or rapid wavelength switching. Fast switching facilitates the ability to follow fast changes in ion concentrations in dual wavelength ratio imaging applications and to monitor changes in the studied system at additional wavelengths.

Features

- DG-4, Xenon 150 or 300 W 340 - 700 nm with 1.2 ms switching time
- AMH, Broad range Metal Halide Source
- LED, High Brightness, instantaneous switching
- PCU controlled synchronisation for Fast λZ imaging

Dual Colour or Dual Camera Solutions

TuCam Dual Camera Adapter

The TuCam is a high performance dual camera adapter, with the ability to fill the field of view of even a large sCMOS sensor and with excellent image quality / registration. The TuCam can be equipped with two identical cameras for simultaneous dual color imaging, or can be used for convenient switch over between cameras with different attributes, say EMCCD and sCMOS.

Features

- Enables simultaneous two-camera exposure with discrimination by wavelength or polarization
- A 100% mirror can be included for easy camera switching
- Independent magnification of 1.0, 1.2, 1.5, 2.0 per camera
- Very reproducible beamsplitter / mirror mounts simplifying exchange and minimizing readjustment

OptoSplit II

If performing simultaneous multi channel imaging, the OptoSplit is a viable alternative to a multi camera set up. The OptoSplit II provides two color image splitting onto a single camera.

Features

- Simple to configure and align
- Easily exchangeable filter sets
- Field splitting for simultaneous two or three color imaging

Three Color and Three Camera Solutions

TuCam and Dual Camera CSU-X1, three camera solution

Utilising the TuCam to give simultaneous two color imaging and using an additional port on the CSU-X1 permits simultaneous three color imaging. Alternatively the TuCam can be used to mount two cameras, of a particular type while having instant access to a different type of camera permanently mounted on the CSU.

OptoSplit III

The OptoSplit III can split the incoming image into three images all of which are imaged onto the same sensor. This has the advantage of being highly cost effective, but considerable sacrifices are made with image resolution.

Application and Technical Notes

The following pages provide examples of how Revolution XD spinning disk confocal systems have been used in a variety of research projects. These articles, based on published data, emphasize the strengths of spinning disk technology in challenging live cell imaging experiments.

We also provide a number of technical notes covering the technology itself, a novel modification, and choosing the right camera.

- **Use Of Spinning Disk Confocal Microscopy To Reveal Chromosome Spindle Attachments**
- **Spinning Disk Confocal Microscopy Helps Reveal Gene Territories In Living Yeast**
- **Spinning Disk Confocal Microscopy**
- **SpiDA - Spinning Disk (Confocal) Anisotropy**
- **Comparing sCMOS With Other Scientific Detectors**

"Our primary focus is to combine electrophysiology with high speed confocal imaging. The XDv provides the required stability for sensitive patch-clamping of neurons whilst imaging thick **living** tissue at the same time."

Dr János Szabadics,
Nikon Microscopy Center,
Institute of Experimental Medicine,
Budapest

ANDOR™ TECHNOLOGY

Application Note

Use Of Spinning Disk Confocal Microscopy To Reveal Chromosome Spindle Attachments

Mitotic cell division relies upon the ability of cells to properly distribute sister chromatids into forming cells. To reliably perform the function cells use internal editing mechanisms to correct inaccurate chromosome / spindle fiber attachments. Paired kinetochores are usually aligned to properly attach spindle fibers and segregate chromatin, however, erroneous and misaligned kinetochores do result. Cells contain specialized editing mechanisms to prevent and correct these misaligned kinetochore pairs, although the exact mechanism is not well understood [1,2] . It is postulated by Tatiana Moutinho-Santos at the Universidade do Porto, Portugal's Instituto de Biologia Molecular e Celular (IBMC) that the presence of POLO kinase is necessary to promote chromosome bi-orientation (termed amphitelic arrangement) and thereby preserve the proper kinetochore alignment.

To better understand its role in regulating kinetochore development, Dr. Moutinho dos Santos studied depleted Drosophila POLO kinase within live cells to substantiate POLO's involvement in the editing functions required to maintain amphitelic kinetochore arrangement. In this case, syntelic arrangement (sister kinetochores attached to microtubules emanating from the same spindle) was studied. Time-lapse analysis of mitosis was performed on Drosophila S2 cells stably expressing CID–mCherry for visualisation of the centromeres, and GFP-α-tubulin for the microtubules. Visualization of kinetochores is demanding due to their small size, low fluorescent signal and brief appearance during cell division. Confocal fluorescence microscopy is often used to image kinetochores, however, even in this environment, visualization remains difficult. The kinetochores are very small (300 nm)[3], close to the lateral resolving capabilities of the light microscope, and exceed the microscope's axial resolving ability. Moreover, imaging fluorescent markers within living cells poses potential phototoxic and photobleaching effects. Intense laser illumination can create phototoxicity issues, especially damaging for living, dividing cells.

Andor's Revolution XD spinning disk confocal microscopy is employed to overcome Dr. Moutinho dos Santo's unique challenge associated with the observation of kinetochores. In this case, four separate factors must be simultaneously addressed to adequately visualize the dynamic cellular processes:

- Speed of acquisition
- Spatial and temporal resolving capability
- The ability to detect very low fluorescence intensity levels
- Management of available fluorescence signal

These themes of spatial, temporal and intensity resolution recur frequently with fluorescence microscopy and are often at odds with experiments involving the observation of cell viability. For example, long camera exposures and extended periods of high intensity illumination generate the phototoxic effects that damage or destroy living cells. Simultaneously, lightly labeled microstructures demand longer exposure to visualize but can be adversely affected by photobleaching. Compounding the issue is the need to combine traditional three-dimensional data with that of time. Finally, it remains necessary to discern signal from background noise and out-of-focus haze.

Speed of acquisition is of particular importance to Dr. Mountinho dos Santos. Control S2 cells exhibit a division cycle of approximately 30 minutes. However, the POLO depleted S2 cells utilized in her experiments showed an arrested period in excess of eight hours. Recording the POLO depleted cell division requires collection of between 7,200 and 12,000 image sets (two fluorochromes imaged every thirty seconds for one to five hours, acquired at 0.5 micron axial steps at up to 20 steps for axial kinetochore resolution). Conventional fluorescence microscopy and laser illumination will permit neither the gentle illumination requirements necessary nor the speed of acquisition to complete this time-sensitive task. For example laser rastering in traditional confocal microscopy requires longer time periods time to collect signal from the sample. Spinning disk confocal systems are used to overcome these traditional barriers and to reveal new insights into molecular kinetochore editing techniques.

Compared to conventional widefield fluorescence systems, the spatial resolution of a spinning disk confocal is superior in both lateral (x and y) and axial (z) dimensions. Through the constant scanning of the pinhole array, samples can be viewed in real-time at high contrast, providing clear images at the diffraction limits of the microscope's optics. This enables the 3D visualization and understanding of the dynamic kinetochore behavior in relation to the microtubules of the spindle. Kinetochore and centromere lateral resolution of 300nm and spindle fiber resolution of 300-500 nm were reported and are detailed in Figure A.

Intensity Resolution

Visualizing the fluorescently labeled kinetochores associated with each centromere places additional demands on the acquisition system. The centromeres and kinetochores under study are primarily visible only during cell division interphase. As the cells pass through prophase, the centromeres have already resolved themselves into Drosophila's typical twelve chromosome pairs. Managing the available light budget during lengthy acquisition necessitates gentle illumination and high resolution capable by spinning disk techniques. The apertures within the spinning disk unit provide these benefits. Because light excites fluorophores only when an aperture is present, phototoxic effects are minimized. While counterintuitive, a reduced light budget does not indicate faint, hard-to-detect objects. Intensity resolution is increased through the spinning disk's exclusion of out

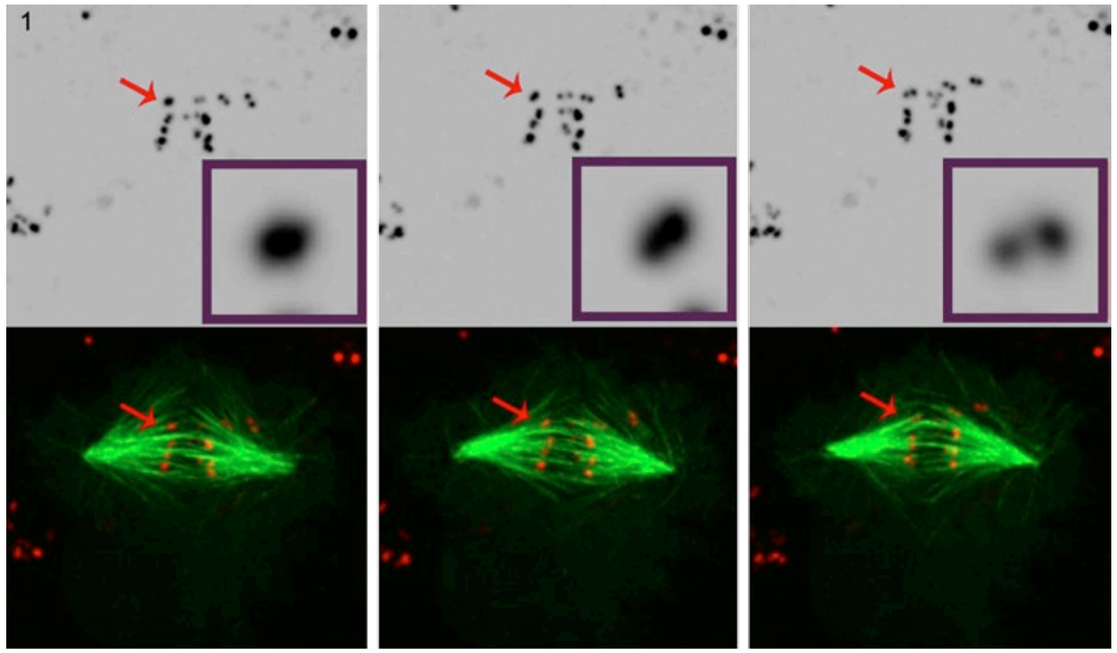


Figure 1. Three Selected still images from a movie of POLO-depleted cells clearly show that, over time, intense centromere/kinetochore signal (red) resolves into a pair of dots, which demonstrates that chromosomes are syntelically attached to spindle microtubules (green). 4D fluorescence microscopy datasets were collected every 30 seconds with 0.5 mm z-steps covering the entire cell volume using a 100°, 1.4 NA plan-apochromatic objective at 25°C with Andor's Revolution spinning disk confocal unit and iXon EMCCD camera, both driven by Andor IQ live-cell imaging software (andor.com).

of focus signal and subsequent signal amplification by an EMCCD camera architecture. This creates the new possibility to generate higher contrast images revealing the development and alignment of punctate objects such as kinetochores.

Conclusions

The use of spinning disk technology in Dr. Mountinho dos Santos live cell application led to an important observation. In the absence of the POLO kinase, Drosophila cells were shown to lack the corrective mechanisms necessary to maintain amphitelic chromosome arrangement. The presence of POLO was shown to provide the right environment for correct centromere architecture, while simultaneously ensuring proper chromosome bi-orientation. Cultured Drosophila cells undergoing mitosis in the absence of POLO kinase chromosomes attach to spindle fibers with syntelic orientation, i.e., with sister kinetochores attached to microtubules that come from a single spindle pole.

This conclusion was drawn by careful analysis of 4D fluorescence microscopy in cells expressing fluorescently labeled centromere/ kinetochore marker and microtubules. (Figure 1).

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Application Note

Spinning Disk Confocal Microscopy Helps Reveal Gene Territories In Living Yeast

It is well understood that the positioning of genes within the cell nucleus is not random and plays a role in certain nuclear functions. It has been postulated that expression for each gene is confined to certain regions within cells, however the understanding for how and why this occurs remains poorly understood. The significance of gene territories within living cells can potentially provide greater understanding for how overall gene expression is affected by gene location within the cell nucleus. This question has been posed by Olivier Gadal and his fellow investigators at the Pasteur Institute in Paris. Dr. Gadal and his colleagues propose that the spatial organization of genome is by no means random, and represents a significant role in genomic transcription, regulation, DNA repair and regulation[1].

To substantiate these observations Dr. Gadal observed the formation of GFP-labeled coregulated galactose and bioregulated biogenesis genes territories in the mCherry-labeled nucleoli of living Saccharomyces cerevisiae yeast cells. Previous observation of gene territories has been performed, however the observations were obtained from fixed cells, and in two dimensions. Dr. Gadal has chosen to observe live cells in a three-dimensional format, which allows more complete and dynamic probabilistic mapping of the sub-nuclear gene territories in question. Central to his observation of cells are three important factors. The first is overcoming the present limitations of fixed cells and the lack of spatial detail required to better predict the probabilistic location of gene territories. Of equal importance is overcoming the physical limits of resolution based upon traditional light microscopy techniques; gene compartment sizes within a eukaryotic nucleus are generally accepted to be only slightly larger than the 500 nanometer axial resolution and 250 nanometer lateral resolution available from typical light microscopes. A final consideration for Dr. Gadal is the need to automatically acquire large numbers of images to more accurately assess gene territories. Stochastic motion within the nucleus requires large populations for study, hence the need for a large number of observations. And because automated methods are employed in the experiment to select nuclei within interphase cells, it is important to have the most highly resolved image sets possible.

Dr. Gadal turned to Andor's Revolution Spinning Disk Confocal Microscope (SDCM) to address current limitations in conventional light microscopy and obtain the necessary high performance required to collect the number of images needed to accurately predict gene territories. In doing so, he addresses the need to maintain the viability of living cells over repeated exposures, collect the required number of observations to make accurate predictions of gene territory areas or regions while simultaneously addressing the resolution requirement needed to automatically select and observe gene loci within their respective domains.

As in many live cell imaging applications, speed of imaging while maintaining highly resolved (both intensity and spatial) objects is the primary requirement. Living cells visualized through fluorescent tags are prone to phototoxic and photobleaching effects. A microscope's optical path provides a certain degree of assistance in managing a limited light budget, but cannot account for all the factors affecting image acquisition. High numerical objective lenses allow more in-focus light to be detected and electron-multiplying CCD cameras effectively amplify that signal while suppressing background noise. The inclusion of a spinning disk assists in the further improvement of signal to noise ratio, while simultaneously creating a more gentle and photostable imaging environment.

Speed of Imaging

Live yeast cells are used to better understand the probabilistic locations of the coregulated galactose and bioregulated biogenesis genes. While previous experiments have determined gene location through fixed yeast cells, a more highly resolved map is the ultimate goal of Dr. Gadal and his colleagues. Stochastic motion within live cells provide that environment, however, the cells must be treated carefully to properly view and map gene locations. The apertures in the spinning disk confocal provide a much gentler imaging environment than traditional widefield or laser scanning techniques, whilst meeting the resolution requirement necessary to visualize sub-resolution detail.

Resolving Sub Resolution Detail

It is commonly accepted that lateral resolution within the light microscope environment is approximately 0.25 micron, with axial resolution commonly accepted to be approximately 0.5 micron. The yeast cell nuclei in Dr. Gadal's experiments are estimated to be 1.0 micron spheres, impressing important dynamic considerations on highly resolved sub-cellular features. It is possible through the use of high numerical aperture objective lenses to restrict the depth of field issues that lead to loss in resolution, however, the addition of the spinning disks pinhole apertures provides a much more highly resolved series of images from which to perform the automated analyses required of the probabilistic gene territory maps.

Collecting Large Amounts of Data

To accurately predict gene locations, it is necessary to collect a large number of yeast nuclei. In this instance approximately 2,000 cells were acquired for each expressed gene and then images automatically processed to include only those in interphase and expressing the gene of interest. In addition to traditional scanning, the third dimension of depth is taken into account. For each population of

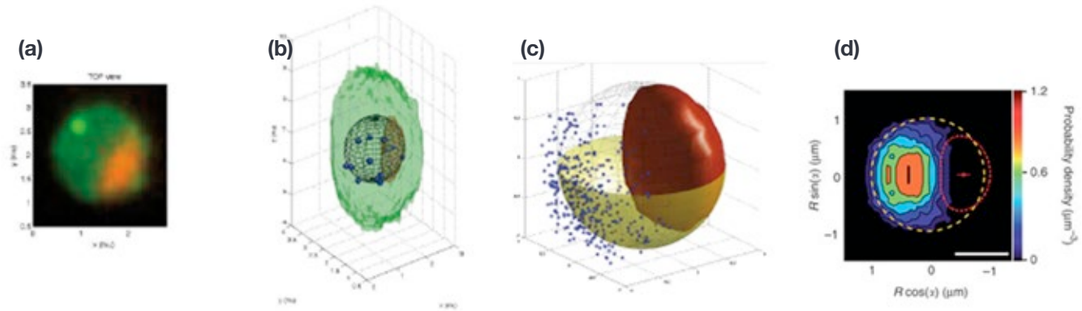

Figure 1a - To substantiate gene expression territories, Fluorescence image sets were acquired via an Andor Revolution Spinning Disk Confocal (Andor, Belfast Ireland) and three dimensional maximum intensity projections were created. Locus and nuclear pores are green and the nucleolus in red. Selection of interphase cells occurred through both manual and automatic detection methods. Manual methods were foirst employed, and then to reduce operator induced variability, automatic detection of interphase nuclei was performed. Using the MatLab-derived routine Nucloc (nucloc.org).

Figure 1b - Resulting regions of interest were processed to first extract 3D loci coordinates, nuclear centers, nucleolar centroids, nuclear envelope ellipsoid and nucleolar volume. The collected data points were further evaluated through custom-developed quality control procedures designed to correct for chromatic shift and to eliminate objects that did fall within measurement parameters used to define the theoretical nucleolar volume extents.

Figure 1c - shows an example 3D view of locus positions (green spheres) obtained from approximately 2,500 nuclei, after nuclear landmark alignment; red spheres indicate nucleolar centroids; yellow hemisphere radius, 1 μm; the red surface shows the 'median' nucleolus.4

Figure 1d - Probability maps for resulting measurements were then created. Nuclei from Fig. 3 are shown as a heat map in Fig 4. The median nuclear envelope is indicated by the dashed circle, the median nucleolus by the dashed red curve, the median location of the nucleolar centroid is indicated by the small red circle4. Additional statistical sampling is used to confirm locations and placements.

cells scanned, 41 axial images are first acquired to visualize the cells' entire volume and then to create the maximum intensity projection used to automatically find nuclei in interphase. Automated nuclei detection requires a stable and repeatable imaging environment. Without it, automation would not be possible, requiring more manual methods, which have been repeatedly shown to reduce experimental objectivity and accuracy.

The work of Dr. Gadal further refines existing research findings that describe the location and extent of gene territories within eukaryotic cell nuclei, and does so over longer time periods than previously reported2. It is revealed through his research that gene territories in question are smaller than anticipated, which in turn constrains the interactions of genes with "each other, the nucleolus or the nuclear envelope." The findings show that positions of genes within the nucleolus are important, as those positions help define whether a gene is activated or repressed. It is through these observations by Dr. Gadal and his colleagues that the role of nuclear architecture is far more important than previously assumed. The role of spinning disk microscopy helps reveal these arrangements and makes these observations possible.

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Technical Note

Spinning Disk Confocal Microscopy

In conventional widefield optical microscopy the specimen is bathed in the light used to excite fluorophores (Figure 1a). The fluorescence emitted by the specimen outside the focal plane of the objective interferes with the resolution of in focus features. As the sample increases in thickness, the ability to capture fine detail above out-of-focus signal becomes increasingly challenging. The confocal principle rejects out-of-focus light through the use of a pinhole, with the added benefit of an improvement in lateral and axial resolution. It is these benefits that make confocal microscopy so popular and why you find one in most core imaging facilities.

As can be seen in Figure 2 (inset), only light (denoted by green) from the right plane passes through the pinhole. Light from the plane above the imaged plane (denoted in red) is focussed above the pinhole so most of the light fails to pass through the pinhole. Conversely light from the plane below the imaged plane (denoted in yellow) focuses before the pinhole and fails to pass through the pinhole. Therefore only light from one plane is in focus to pass through the pinhole or as the name suggest 'confocal'. A similar principal applies to a pinhole camera which uses a pinhole to obtain crisp images without a lens.

Confocal microscopy is now a routinely used optical technique across a wide range of biological sciences, from plant science to mammalian models. Its use is popular because it has several benefits over conventional widefield microscopy, namely in its ability to remove out-of-focus signal, capture information from a reduced depth of focus, image discreet optical sections in thick samples and so create high contrast 3D image sets.

The most common confocal technology is that of confocal laser scanning microscopy (CLSM). In this format the sample is illuminated by a single point of light from a laser (Figure 1b). The laser beam is scanned point by point in a raster pattern and signal is detected sequentially from each point by a photomultiplier tube until an entire image is created. With CLSM, there is a trade-off between image resolution and speed. If the array consists of a 512 x 512 pixel array and each point is illuminated for 1 microsecond, then each scan will take about 262 milliseconds. The signal from each point must be acquired in that 1 µs and there is time 'skew' of 0.26 second between the first and last points in the scan. To compensate for the brief illumination of each pixel, an intense laser beam is required, and if the specimen is dynamic the time skew can lead to errors in observation.

Spinning disk confocal laser microscopy (SDCLM) overcomes this problem by exploiting the multiplex principle. Figure 1c illustrates how the sample is illuminated, and so light detected, at multiple points simultaneously. This was originally proven by Felgett in spectroscopy and shows that using parallel detection delivers enhanced sensitivity [1]. A publication by Wang [2] provides a quantitative comparison of point and disk scanning systems for imaging live-cell specimens.

Figure 2 shows how the dual spinning disk confocal laser scanner operates. Unlike a conventional laser-scanning microscope, where a narrow laser beam sequentially scans the sample, in SDCLM an expanded beam illuminates an array of microlenses arranged on a (collector) disk. Each microlens has an associated pinhole laterally co-aligned on a second (pinhole) disk and axially positioned at the focal plane of the microlenses.
The disks are fixed to a common shaft that is driven at high speed by an electric motor. When the disks spin, and the scanner is coupled to a microscope with the pinhole disk located in its primary image plane, an array of focused laser beams scan across the specimen [3]. Yokogawa realised the benefit of this approach and created their CSU spinning disk confocal unit [4] which is now widely used for confocal live cell imaging.

The pinholes (and microlenses) are arranged in a pattern [4], which scans a field of view defined by the array aperture size and the microscope objective magnification. The scanning laser beams excite fluorescent labels in the specimen. Fluorescence emission will be most intense where this array is focused – the focal plane. Some fraction of this light will return along the excitation path where it will be preferentially selected by the same 'confocal' pinholes. A dichroic mirror, which reflects emission wavelengths, is located between the two disks. This separates the laser emission from any excitation light reflected or scattered from the microscope optics. The geometry of the emission path results in a confocal fluorescence signal with extremely low background noise.

A further limitation in conventional CSLM is the use of photomultiplier tubes (PMTs) whose quantum efficiency (QE, the probability of converting a photon to an electron) is rather low – typically 30-40%. In contrast the SDCLM technique uses a camera as a detector that can have a very high QE; e.g. an iXon EMCCD has a peak QE of more than 90%, making it a near-perfect detector. This combination of the Yokogawa dual spinning disk and high QE detector delivers a confocal instrument that can run at high speed and with unequalled signal-to-noise (SNR).

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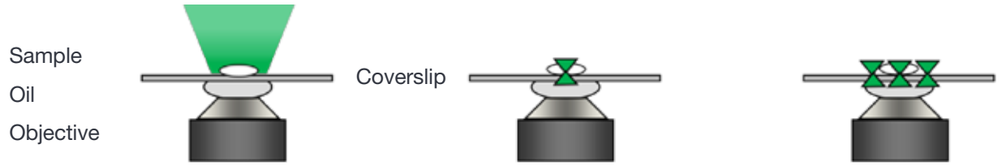

Figure 1. Schematic diagram illustrating the pattern of illumination for conventional widefield, laser scanning, and spinning disk microscopy. In widefield microscopy (a) the sample is broadly illuminated with excitation light with limited focus of its energy dictated through the numerical aperture and focal depth of the objective. Laser scanning confocal microscopy (b) focuses a single point of laser light through a small aperture (pinhole) and scans sequentially across the sample point by point. Spinning disk confocal microscopy (c) illuminates the sample with a rotating pattern of 1,000's of pinholes for complete simultaneous confocal illumination.

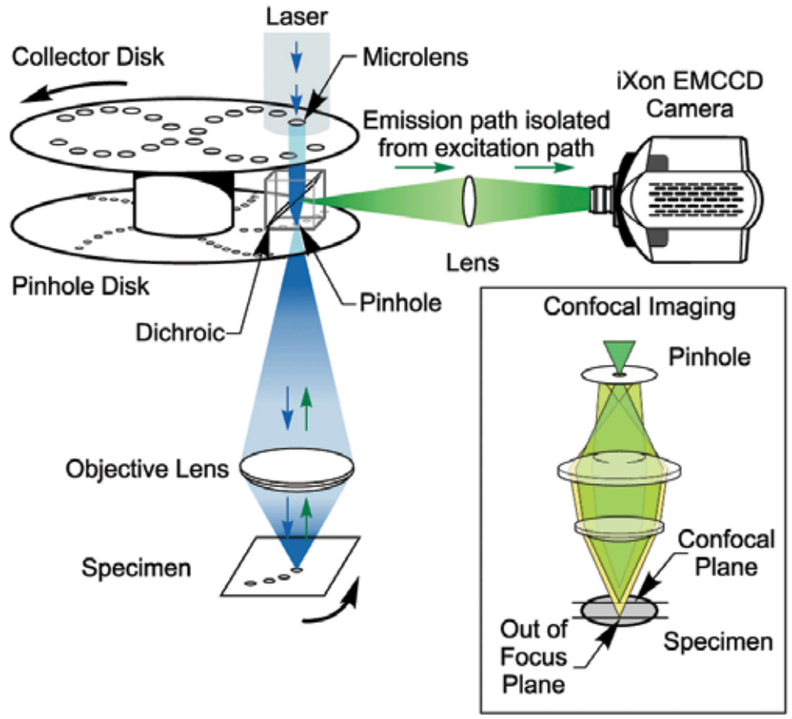

Figure 2. Dual disk arrangement of the Yokogawa CSU-X. In addition to the fundamental disk containing an array of pinholes there is a second collector disk with a matching pattern of microlenses. The microlenses focus the excitation light with greater efficiency onto the imaging pinholes so increasing excitation throughput from a limited ~2% up to a functional 70%. This technology improvement, in combination with using an electron multiplying CCD detector, results in spinning disk technology being the ideal solution for fast live cell confocal imaging.

Technical Note

SpiDA - Spinning Disk (Confocal) Anisotropy

Anisotropy imaging can be used to elucidate dynamic molecular organization in living cell studies. For optimum speed and dynamic range, laser spinning disk confocal imaging is the best tool currently available. We have overcome a limitation in the standard CSU scan head to enable use for real time confocal imaging of fluorescence anisotropy.

1. Introduction

The technique of fluorescence anisotropy is well-known in fluorescence spectroscopy and biochemical assays (Lakowicz 2006) and it has been used in epi-fluorescence microscopy in living cells (Lidke et al 2003, Sharma et al 2004, Goswami et al 2008) and even in super-resolution imaging (Gould et al 2008). However, when implemented in point scanning confocal instruments with live cell specimens it has suffered from photo-bleaching, low frame rates and temporal skew resulting from sequential point scanning.

The CSU (Ichihara et al 1996), manufactured by Yokogawa Electric Corporation, is the leading spinning disk confocal scanner on the market today and finds widespread application in live cell confocal imaging. The various CSU models provide scan rates up to 2000 frames per second, with good confocality (~1 um FWHM) and low background across the visible range (400-650 nm). Unfortunately, the CSU cannot be used in its native form for anisotropy studies because its excitation path degrades the polarization of laser light. We have overcome this limitation and we present here a spinning disk confocal anisotropy system (SpiDA) which exploits simultaneous dual camera detection of orthogonal emission polarizations to provide high definition spatio-temporal anisotropy imaging.

We have worked with Dr Satyajit Mayor and his team at NCBS in Bangalore, to bring this solution to fruition and a Revolution XD systems now resides is in his laboratory, where it is heavily used for confocal anisotropy studies in living cells.

2. Anisotropy and Homo-FRET

Isotropic is derived from the Greek and means quite simply “equal in all directions”. While anisotropic means not equal in all directions. Fluorescence anisotropy measurements are based on the principle of photo-selective excitation by polarized light.

Fluorescent molecules preferentially absorb photons whose electric vectors (polarization) are parallel to their absorption (transition) electric dipole. The dipole has a defined orientation with respect to the molecular axis. Thus, when polarized light is incident on a population of molecules it is absorbed preferentially according to orientation. Further, the resulting fluorescence emission is also aligned relative to the molecular axis and the relative angle between absorption and emission polarization determines anisotropy.

Fluorescence anisotropy, r is defined as follows:

$$r(t) = (I_p(t) - I_s(t))/ (I_p(t) + 2\, I_s(t))$$

where $I_p(t)$ is the fluorescence intensity parallel to excitation and $I_s(t)$ is fluorescence intensity perpendicular to excitation (Lackowicz 2006).

As the notation indicates fluorescence anisotropy, r is a function of time and decays with a relaxation time, Trot. The relaxation time is a measure of rotational diffusion which occurs during the lifetime of the excited state (typically 1-10 ns). In fluids molecular rotation can take place in a few tens to hundreds of ps and as result little anisotropy is observed. The rotational diffusion rates of larger molecules, such as proteins, are of the same order as fluorescence lifetimes and therefore anisotropy is sensitive to factors affecting these rates.

Anisotropy can therefore be used as an indicator of the state of biological macro-molecules within the cell and its membranes, including molecular size, aggregation and binding state (Lidke et al 2003).

When excited fluorescence molecules (donors) come close enough to engage in dipole interactions (0.3-0.5 nm) with unexcited fluorescence molecules (acceptors), an effect known as resonant energy transfer (RET) can occur. Provided the excitation spectrum of the acceptor overlaps the emission spectrum of the donor, this can result in transfer of energy from donor to acceptor and is non-radiative (no photon is emitted). The newly excited molecule can now emit a photon, but its polarization will depend on its own orientation. When averaged over an ensemble, the result of RET is loss of polarization and reduction in anisotropy.

When the RET interactions happen between fluorescent molecules of the same type the process is known as “HomoFRET”, and can be used to monitor molecular interactions and binding states. Varma and Mayor (1998) used anisotropy to monitor HomoFRET interactions in so-called lipid rafts and GPI-anchored proteins, which are located in the plasma membrane of living cells. These structures are important in key cellular processes and Mayor and co-workers have made substantial contributions to understanding these structures and their function using anisotropy imaging.

Figure 1 provides a visual summary of the principles of selective excitation and how rotational diffusion and HomoFRET affect polarization and anisotropy.

3. Polarization and the CSU

Polarization measurements in the Yokogawa CSU products shipped from the manufacturer show that laser polarization is degraded in the excitation optics of the instrument (Table 1). This was common in 5 different units tested and included CSU-10, 22 and X1 models. A low extinction ratio precludes use of an unmodified CSU for anisotropy imaging. In contrast the emission path was found to maintain polarization with high fidelity enabling confocal detection of the polarization state in all units tested to date.

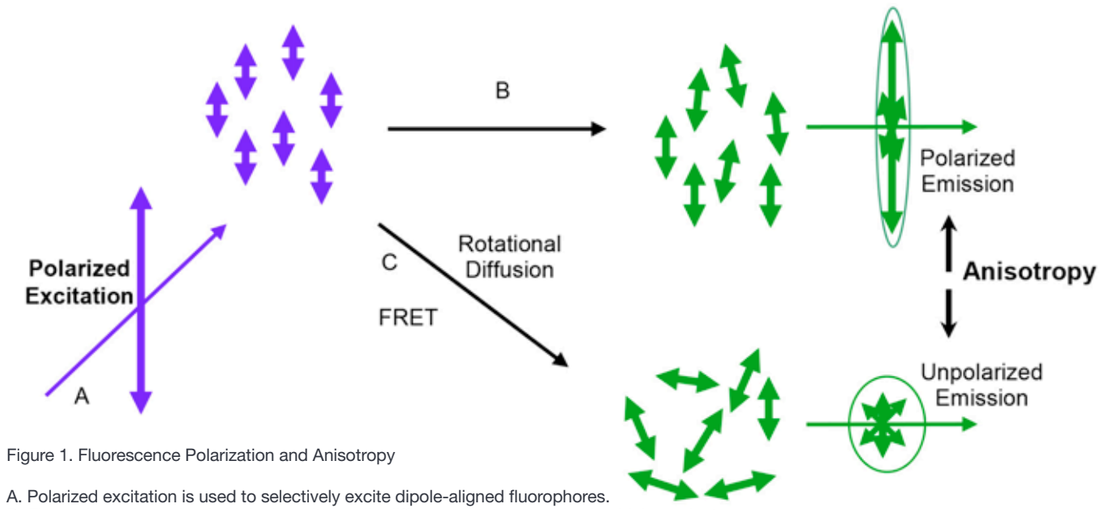

Figure 1. Fluorescence Polarization and Anisotropy

A. Polarized excitation is used to selectively excite dipole-aligned fluorophores.
B. Fluorophores bound or in high viscosity media diffuse or rotate more slowly - higher anisotropy
C. Rotational diffusion or resonance energy transfer reduces polarization – lower anisotropy

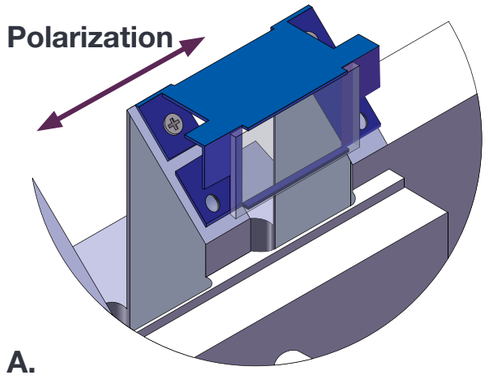

Figure 2. Key components of the spinning disk confocal anisotropy imaging system are highlighted.
A. Shows a schematic of the CSU-P polarizer mounted on custom-designed carrier. The component is motorized to allow introduction into the optical path or removal for convenient switching of operational modes. B. TuCam high performance dual camera adapter with two iXon EMCCD cameras attached. TuCam is designed to deliver alignment stability and low differential distortion (<1%). These features ensure that calibration tables for sub-pixel image registration do not require frequent updates to deliver precise anisotropy computation.

Hardware configuration	Ext Coefficient
ALC + SMP/PM Fiber	147
ALC + Fiber +CSUX1	16
ALC + Fiber + CSU-P – see text	125

Table 1 - Polarization results from laser engine and SM/PM fiber (before the CSU-X1), standard CSU-X1scanner and the Andor-modified CSUIX1 polarization solution (CSU-P). Standard CSU-scanners degrade input laser polarization as this table shows.

We found that a good solution to the polarization problem is to integrate a high performance polarizer into the optical path of the CSU at a position which affects the excitation (laser) light, but not the detected fluorescence, whose polarization contains the useful information. A custom carrier was designed for the polarizer as shown in Figure 2a and this is mounted on a motorized drive for insertion and removal under computer control. In this way conventional intensity imaging can be re-selected without compromising sensitivity.

4. Confocal Dual Camera Anisotropy Imaging

The epi-illumination system described in Varma and Mayor (1998) was limited for dynamic studies because it used a single camera and sequential detection of p and s polarization images using a filter wheel. This approach introduces a time-skew between the s and p channels. To overcome this Mayor’s group constructed a dual camera solution (Goswami et al 2009) and access to dynamic data was extended. Post acquisition image processing is required to achieve pixel alignment of the two images prior to the calculation of anisotropy.

However, a remaining problem with such a system is that emission

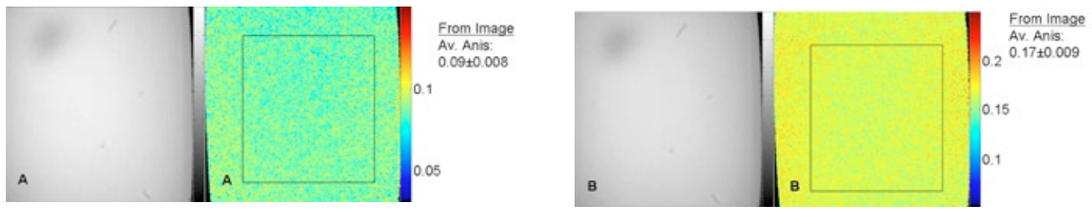

Figure 3. Calibration images are created by imaging FITC-loaded glycerol solutions of different concentrations. Higher glycerol concentration slows rotational diffusion rate to a greater extent, leading to higher anisotropy. A. Pseudo-color anisotropy image of 50% glycerol solution with mean isotropy of 0.09 ± 0.008 (SD). B. Pseudo-color anisotropy image of 70% glycerol solution with mean isotropy of 0.17 ± 0.009 (SD).

from microscopic polarization domains is masked by out of focus fluorescence. Hence resolution, dynamic range and signal to background ratio of anisotropy are all compromised. The SpiDA system greatly reduces these effects by rejecting out of focus fluorescence, allowing anisotropy domains to be monitored in greater detail than ever before.

To achieve maximal temporal resolution and full field of view, SpiDA employs TuCam, our dual camera adapter. TuCam is a third generation adapter optimized for throughput, distortion and ease of alignment and enables simultaneous detection of p and s polarizations onto back-illuminated EMCCD cameras i.e. iXon EMCCD - see Figure 2b.

An image quality polarizing beam splitter is a critical component of the system: it must be flat and mounted with minimal stress. We utilize laser quality components with a surface flatness of $<\lambda$ per inch and radius of curvature $\geq$ 30m. This minimizes distortions and lensing effects which can lead to focus error across the field of view. The beam splitter is mounted in a kinematic assembly to provide precision of adjustment and stability.

Mechanical stability is the most critical quality of an image splitter to ensure robust and repeatable measurement. Drift or creep leads to registration errors. Even though image registration to sub-pixel precision requires image processing in software, this process is driven by calibration whose temporal repeatability depends on the physical design. We have optimized image registration in our own iQ software, but an open source solution is also available in ImageJ (http://rsbweb.nih.gov/ij/).

The iXon cameras deliver high signal to noise anisotropy imaging with exposures in the 10-100 ms range. This provides a dynamic imaging tool for protein-protein interactions and microstructure modulation during events such as endocytosis and vesicle recycling. Figures 4 and 5 show example calibration data and live anisotropy images respectively.

5. Conclusions

This technical note describes the Andor solution for real time confocal anisotropy imaging, which has been developed to overcome inherent constraints in the CSU scan head for polarized excitation of fluorescence. SpiDA utilizes a high performance image splitting dual camera adapter, TuCam and ultra-sensitive EMCCD cameras to deliver high contrast dynamic anisotropy data for live cell studies. We highlight cooperation with a scientific research group whose vision had not been realized previously. This process has now delivered a solution which is generally available to the wider research community as the SpiDA option for Revolution XD.

We take this opportunity to thank Dr Satyajit Mayor and his team at NCBS for their support, feedback and collaboration during this project.

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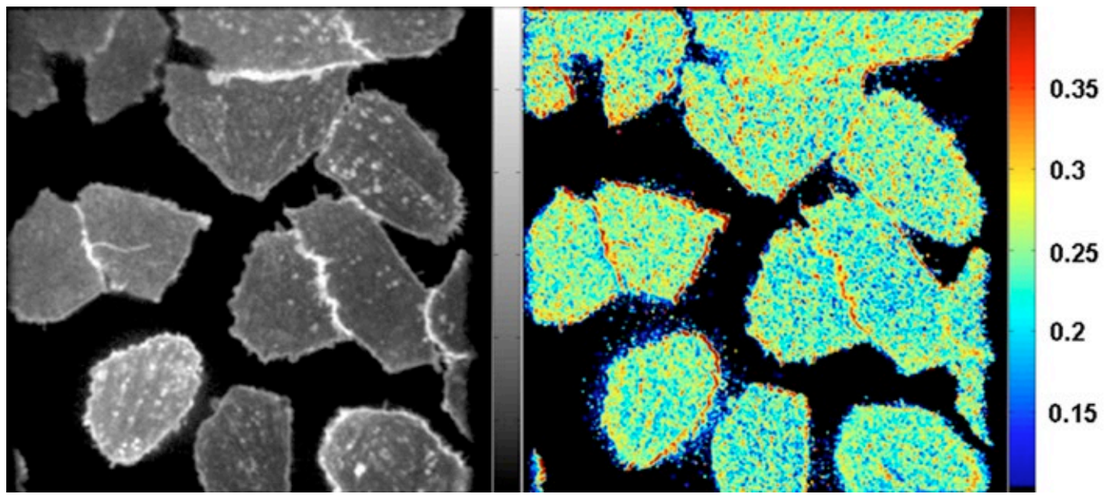


Figure 4. Anisotropy images from live cells after registration and pixel computation. Hot spots in anisotropy are indicative of tethered or bound fluorophores and reflect microstructure domains in the plasma membrane. Image courtesy of Mayor Lab, NCBS, Bangalore. Note GG8 cells are a CHO variant devoid of transferring receptor (Tfr) stably transfected with human Tfr and GFP-GPI (Sabharanjak et al 2002).

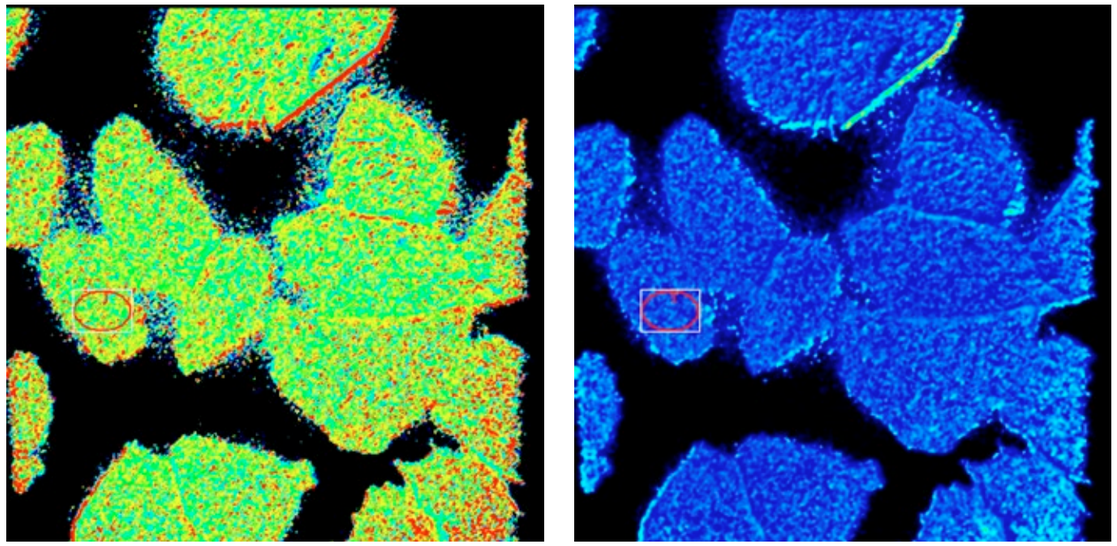


Figure 5. Images showing anisotropy of cultured GG8 cells in single confocal optical sections at or close to the plasma membrane. A. Domains of GFP labelled lipo-proteins showing localized high anisotropy (mean r = 0.29) are clearly highlighted in yellow and red in the pseudo color mapping. These domains have been shown (Sharma et al 2004) to be associated with cholesterol. B. The same field in the specimen imaged after treatment with saponin, a natural “soapy” product of some plants (e.g. soapwort), which forms complexes with cholesterol in the plasma membrane. The impact of this on lipo-protein domain anisotropy is dramatic (mean r =0.17). Image courtesy of Mayor Lab, NCBS, Bangalore.

Technical Note

Comparing sCMOS With Other Scientific Detectors

sCMOS technology is unique in its ability to overcome many of the mutual exclusivities that have marred other scientific detector technologies, resulting in an imaging detector that simultaneously optimizes a range of important performance parameters whilst maintaining Snapshot exposure capability.

Part 1 - Current scientific imagers: Interline CCD and EMCCD

Many scientific imaging applications demand multi-megapixel focal plane sensors that can operate with very high sensitivity and wide dynamic range. Furthermore, it is often desirable that these sensors are capable of delivering rapid frame rates in order to capture dynamic events with high temporal resolution. Often there is a strong element of mutual exclusivity in these demands. For example, it is feasible for CCDs to achieve less than 3 electrons rms readout noise, but due to the serial readout nature of conventional CCDs, this performance comes at the expense of frame rate. This is especially true when the sensor has several megapixels of resolution. Conversely, when CCDs are pushed to faster frame rates, resolution and field of view are sacrificed (i.e. fewer pixels per frame to read out) or read noise and dynamic range suffer.

By way of illustration, consider one of the most popular, high-performance front-illuminated scientific CCD technologies on the market today – the Interline CCD. These devices are capable of reading out at 20 megapixel/s per output port with a respectable read noise of only 5 to 6 electrons rms. At this readout speed a single port 1.4 megapixel sensor can achieve 11 fps. Use of microlenses ensures that most of the incident photons are directed away from the Interline metal shield and onto the active silicon area for each pixel, resulting in peak QE greater than 60%. High performance combined with low cost has made the Interline CCD a very popular choice for applications such as fluorescence cell microscopy, luminescence detection and machine vision. However, even 5 to 6 e^- noise is too high for many low light scientific applications. For example, when imaging the dynamics of living cells, there is a need to limit the amount of fluorescence excitation light, such that both cell phototoxicity and photobleaching of the fluorescent dyes is minimized. The use of lower power excitation results in a proportionally lower fluorescent emission signal from the cell. Also dynamic imaging yields shorter exposure times per frame, thus fewer photons per frame. Ultra low light conditions mean that the read noise floor can often become the dominant detection limit, seriously compromising the overall signal-to-noise ratio (SNR) and hence the ability to contrast fine structural features within the cell. As such, the inability to maintain low noise at faster readout speeds limits the overall flexibility of the Interline CCD camera.

The Electron Multiplying CCD (EMCCD) was introduced into the market by Andor in 2000 and represents a significant leap forward in addressing the mutual exclusivity of speed and noise as discussed above. EMCCD cameras employ an on-chip amplification mechanism called 'Impact Ionization' that multiplies the photoelectrons that are generated in the silicon. As such, the signal from a single photon event can be amplified above the read noise floor, even at fast, multi-MHz readout speeds. Importantly, this renders the EMCCD capable of single photon sensitivity at fast frame rates (e.g. 34 fps with a 512 x 512 array). This attribute has rapidly gained recognition for EMCCD technology in demanding low light measurements, such as single molecule detection.

However, despite the sensitivity under extremely low light conditions, there are a few remaining drawbacks of EMCCD technology. The amplification mechanism required to reduce the effective read noise to < 1e^-, also induces an additional noise source called multiplicative noise. This effectively increases the shot noise of the signal by a factor of 1.41, which is manifested as an increase in the pixel to pixel and frame to frame variability of low light signals. The net effect of multiplicative noise is that the acquired image has a diminished signal-to-noise ratio, to an extent that the QE of the sensor can be thought to have been effectively reduced by a factor of two. For example, a QE-enhanced back-illuminated EMCCD with 90% QE has effectively 45% QE when the effects of multiplicative noise are considered. Dynamic range limitations of EMCCDs must also be considered. It is possible to achieve respectably high dynamic range with a large pixel (13 to 16 µm pixel size) EMCCD, but only at slow readout speeds. As such, higher dynamic range can only be reached at slower frame rates (or with reduced array size) with modest EM gain settings. Application of higher EM gain settings results in the dynamic range being depleted further. Sensor cost of EMCCD technology is an additional consideration, along with the practical restriction on resolution and field of view that accompanies sensor cost. Presently, the largest commercially available EMCCD sensor is a back-illuminated 1024 x 1024 pixel device with 13 µm pixel pitch, representing a 13.3 x 13.3 mm sensor area. This already carries a significant cost premium, making further expansion to multi-megapixel devices a costly proposition.

Array Size (H x V)	Rolling Shutter mode (fps)	Global (Snapshot) Shutter mode (fps)
2560 x 2160 (full frame)	100	50
2064 x 2048 (4 megapixel)	104	52
1392 x 1040 (1.4 megapixel)	204	100
512 x 512	412	200
128 x 128	1,616	711

Table 1 - Frame rate vs sub-window size; Rolling and Global Shutter readout modes. N.B. Same sub-window frame rates apply when using full horizontal width with the vertical heights indicated (see body text for further detail).

Part 2 - sCMOS: Circumventing the trade-offs

Scientific CMOS (sCMOS) technology is based on a new generation of CMOS design and process technology. This device type carries an advanced set of performance features that renders it entirely suitable to high fidelity, quantitative scientific measurement. sCMOS can be considered unique in its ability to simultaneously deliver on many key performance parameters, overcoming the 'mutual exclusivity' that was earlier discussed in relation to current scientific imaging technology standards, and eradicating the performance drawbacks that have traditionally been associated with conventional CMOS imagers.

The 5.5 megapixel sensor offers a large field of view and high resolution, without compromising read noise or frame rate. The read noise in itself is exceptional, even when compared to the highest performance CCDs. Not even slow-scan CCDs are capable of this level of read noise performance. High-resolution, slow-scan CCDs are typically characterized by seconds per frame rather than frames per second. The fact that the sCMOS device can achieve 1 electron rms read noise while reading out 5.5 megapixels at 30 fps renders it truly extraordinary in the market. Furthermore, the sensor is capable of achieving 100 full fps with a read noise 1.3 electrons rms.
By way of comparison, the lowest noise Interline CCD reading out only 1.4 megapixels at ~ 16 fps would do so with ~ 10 electrons read noise.

Greater speed is available through selection of 'region of interest' sub-windows, such that the field of view can be traded off to achieve extreme temporal resolution. Table 1 shows frame rates that can be expected from a series of sub-window sizes, in both rolling shutter and global shutter modes of operation (the distinction between these two modes is explained later in this paper). Note that each of the sub-windows can be expanded to full width in the horizontal direction and still maintain the same indicated frame rate. For example, both 1390 x 1024 and 2560 x 1024 sub-window sizes each offer 220 fps in rolling shutter mode. This is important information for some applications that can take advantage of an elongated (letter box shape) region of interest.

The low noise readout is complemented by a high dynamic range of 30,000:1. Usually, for CCDs or EMCCDs to reach their highest dynamic range values, there needs to be a significant compromise in readout speed, yet sCMOS can achieve this value while delivering 30 fps. Furthermore, the architecture of sCMOS allows for high dynamic range by offering a large well depth, despite the small pixel size. By way of comparison, a 1.4 megapixel Interline with similarly small pixels achieves only ~1,800:1 dynamic range at 16 fps.

Part 3 - Comparing sCMOS to other leading scientific imaging technologies

A short comparative overview of sCMOS is provided in Table 2. For the purposes of this exercise, we limited the comparison to Interline CCD and EMCCD technologies, given their popularity across the range of scientific imaging applications. Interline CCDs are typified by a choice of 1.4 megapixel or 4 megapixel sensors. The most popular EMCCD sensors are 0.25 or 1 megapixel, typically offering up to 30 fps.

It is apparent that across most parameters, sCMOS presents a distinct performance advantage, notably in terms of noise, speed, dynamic range and field of view/resolution. Importantly, these advantages are met largely without compromise. Whilst the read noise of sCMOS is very low, EMCCD technology still maintains the distinct advantage of being able to multiply the input signal above the read noise floor, thus rendering it negligible (<1 e^-). The majority of EMCCD cameras purchased at this time are also of back-illuminated, having ~ 90% QE max, which also feeds into the sensitivity comparison. For this reason, EMCCD technology will still hold firm in extreme low-light applications that require this level of raw sensitivity, and are willing to sacrifice on the enhanced resolution, field of view, dynamic range and frame rate that sCMOS can offer.

Parameter	Neo sCMOS	Interline CCD	EMCCD
Sensor Format	5.5 megapixel	1.4 to 4 megapixel	0.25 to 1 megapixel
Pixel Size	6.5 µm	6.45 to 7.4µm	8 to 16 µm
Read Noise	1 e^- @ 30 fps 1.3 e^- @ 100 fps	4 -10 e^-	< 1e^- (with EM gain)
Full Frame Rate (max.)	Sustained: >30 fps full frame Burst: 100 fps full frame	3 to 16 fps	~ 30 fps
Quantum Efficiency (QE)	57%	60%	90% 'back-illuminated' 65 % 'virtual phase'
Dynamic Range	30,000:1 (@ 30 fps)	~ 3,000:1 (@ 11 fps)	8,500:1 (@ 30 fps with low EM gain)
Multiplicative Noise	none	none	1.41x with EM gain (effectively halves the QE)

Table 2 - Comparison summary of typically specifications of Interline CCD and EMCCD technologies compared to sCMOS technology.

Figures 1 to 4 show the results of head to head sensitivity comparisons, pitching a prototype 5.5 megapixel sCMOS camera against a 1.4 megapixel Interline CCD device, and also against 1 megapixel back-illuminated EMCCD. The sCMOS was set up to image at 560 MHz, this readout speed capable of achieving 100 full fps, with only 1.3 electrons read noise. The Interline CCD camera, an Andor 'Clara', was read out at 20 MHz, achieving 11 fps with 5 electrons read noise (representing extreme optimization of this sensor at this speed). The EMCCD camera, an Andor iXon 888, was read out at 10 MHz with x300 EM gain amplification, achieving 9 fps with 0.15 electrons effective read noise. Low light imaging conditions were created using (a) a light tight imaging rig, fitted with a diffuse, intensity-variable 622 nm LED light source and mask overlay (consisting either an array of holes or a USAF resolution chart); (b) both confocal spinning disk and conventional widefield fluorescence microscopes, imaging fixed bovine epithelial cells labelled with BODIPY FL (emission max. ~ 510nm).

The LED rig proved excellent for comparing sensitivity under extreme low light conditions, using two low light intensity settings; 10 photons/6.5 µm and 32 photons/6.5 µm. The SNR superiority of sCMOS over even well-optimized Interline CCD technology can clearly be observed, manifest as better contrast of signal against a less noisy read noise background, resulting also in better resolution of features. However, comparison of the two technologies against back-illuminated EMCCD (Figure 2) at the weakest LED setting, showed that the < 1 electron noise floor and higher QE of the EMCCD resulted in notably superior contrast of the weak signal from the noise floor.

Figures 3 and 4 show clear differences in low light signal contrast between sCMOS and Interline cameras, employed on both spinning disk and widefield fluorescence microscopy set-ups. Again the contrast difference arises from the read noise difference between the two technologies.

To further supplement the relative sensitivity performance of these imaging technologies, theoretical SNR plots that are representative of these three technologies are given in Figures 5 and 6. For this comparative exercise, specifications were used that reflect the most sensitive Interline CCD and back-illuminated EMCCD sensors on the market today.

Figure 5 shows how the SNR of sCMOS compares to that of Interline CCD across a range of photon fluxes (i.e. incident light intensities). The pixel size differences between the two sensor types is negligible, thus there is no need to further correct for differing areas of light collection per pixel. The sensitivity differences between the two technology types is reflected in the marked variance between the respective SNR curves at low to moderate photon fluxes. At higher photon fluxes, there is no 'cross-over' point between sCMOS and Interline CCD curves. Similar QE and pixel size ensures that the Interline CCD will never surpass the SNR performance of sCMOS. In fact, due to the significantly lower read noise, the sCMOS exhibits markedly better signal-to-noise than the Interline CCD until several hundred photons/pixel at which point the two curves merge as the read noise of both sensors becomes negligible compared to the shot noise.

Figure 6 shows SNR plots that compare sCMOS and Interline CCD sensors with that of back-illuminated EMCCD sensors. The plot assumes that all three sensors have the same pixel size, which could effectively be the case if the ~ 6.5 µm pixels of both the sCMOS and Interline CCD sensors were to be operated with 2 x 2 pixel binning, to equal a 13 µm EMCCD pixel (representative of a popular back-illuminated EMCCD sensor on the market). As such, the photon flux is presented in terms of photons per 13 µm pixel (or 2 x 2 binned super-pixel), relating to an actual pixel area of 169 μm^2. There are two notable cross-over points of interest, relating to where the EMCCD S/N curve crosses both the sCMOS and Interline CCD curves, which occur at photon flux values of ~ 55 photons/pixel and ~ 225 photons/pixel, respectively. At photon fluxes lower than these cross-over points the EMCCD delivers better S/N ratio, and worse S/N ratio at higher photon fluxes. The reason that a back-illuminated EMCCD with negligible read noise does not exhibit higher S/N right throughout the photon flux scale, is due to the multiplicative noise of the EMCCD plot (which effectively increases the shot noise).

Figures 7 and 8 show widefield fluorescence microscope images, taken using x60 and x100 magnifications respectively, comparing 5.5 megapixel sCMOS to 1.4 megapixel Interline CCD technology. Each clearly reveal the markedly larger field of view capability of the 5.5 megapixel sCMOS sensor. Since each sensor type has ~ 6.5 µm pixel pitch, allowing for adequate NyQuist oversampling at the diffraction limit, it is unsurprising that each show virtually identical resolution of fine intracellular structure under brighter conditions, as shown in Figure 8. At low photon fluxes however, typified in figures 3 and 4, the higher read noise of the Interline device results in greater sacrifice in resolution and contrast. This is a decisive point for live cell measurements, which often necessitate the use of low illumination energies.

Conclusion

After several decades of technology maturation, we have now reached a 'leap forward' point, where we can confidently claim that the next significant wave of advancement in high-performance scientific imaging capability has come from the CMOS imaging sensor technology stable. Scientific CMOS (sCMOS) technology stands to gain widespread recognition across a broad gamut of demanding imaging applications, due to its distinctive ability to simultaneously deliver extremely low noise, fast frame rates, wide dynamic range, high quantum efficiency, high resolution and a large field of view. Comparisons with other current 'gold standard' scientific image detector technologies show that the CIS 2051 sCMOS sensor, optimized in the Andor Neo camera, out-performs even high-performing interline CCD camera in most key specifications whilst maintaining a Snapshot exposure mode for broader application flexibility.

For extremely low light applications that require absolute raw sensitivity at respectably fast frame rates, a high performance back-illuminated EMCCD camera (present in the Andor iXon range) maintains an application advantage.

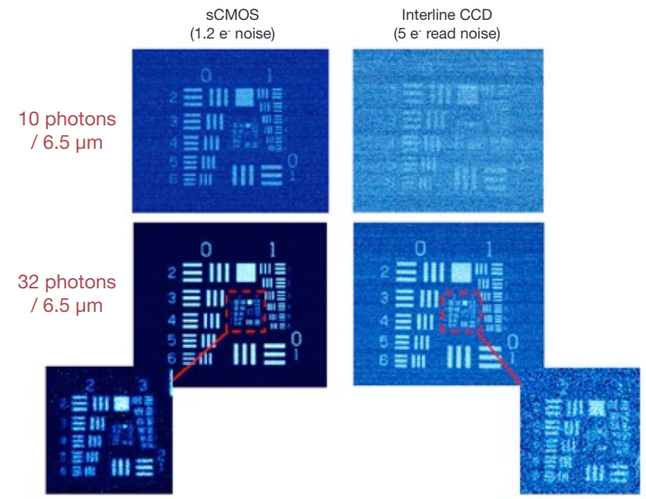


Figure 1 - Comparative low light images of a USAF resolution chart, showing Andor sCMOS (1.3 electrons read noise @ 560 MHz) vs Interline CCD (5 electrons read noise @ 20 MHz), under the two lowest LED settings.

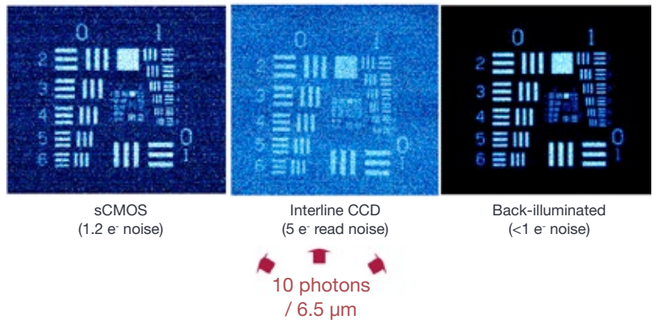


Figure 2 - Comparative low light images taken with Andor sCMOS (1.3 electrons read noise @ 560 MHz) vs Interline CCD (5 electrons read noise @ 20 MHz) vs back-illuminated EMCCD (< 1 e⁻ read noise), under extremely low light conditions (10 photons / 6.5 µm setting). sCMOS and Interline CCD were 2 x 2 binned in order to have the same effective pixel pitch (and light collection area per pixel) as the 13 µm pixel of the EMCCD sensor.

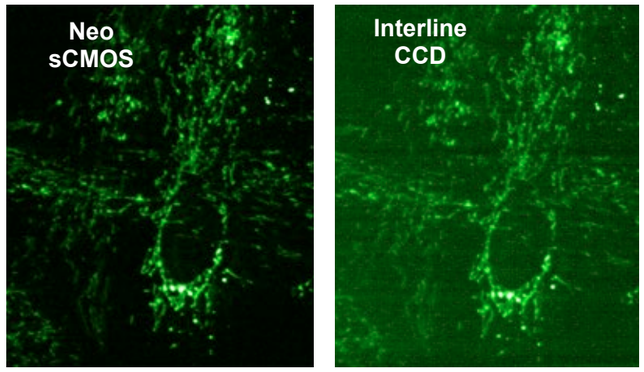
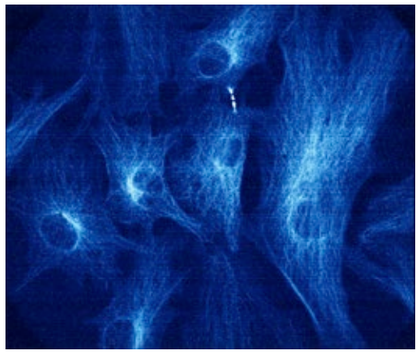


Figure 3 - Comparative low light images taken with Andor sCMOS (1.3 electrons read noise @ 560 MHz) vs Interline CCD (5 electrons read noise @ 20 MHz) of fluorescently labelled fixed cell using a CSU-X spinning disk confocal microscope (x60 oil objective), each 100 ms exposure, same laser power, displayed with same relative intensity scaling. Note, the field of view is limited by the aperture size of the CSU-X, which is matched to the 1.4 megapixel Interline sensor.

sCMOS (1.2 e⁻ noise)



Interline CCD (5 e⁻ read noise)

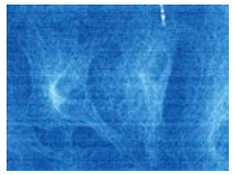


Figure 4 - Comparative low light fluorescence microscopy images taken with Andor sCMOS (1.3 e⁻ @ 560 MHz) vs Interline CCD (5 e⁻ @ 20 MHz) under low light conditions, typical of those employed in dynamic live cell imaging. ND filters on a widefield fluorescence microscope were used to reduce light levels relative to the read noise floor.

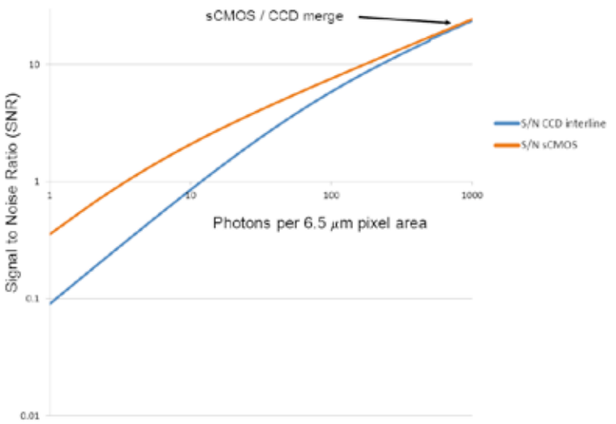


Figure 5 - Theoretical Signal to Noise plot comparisons for sCMOS vs Interline CCD sensors. Photon flux (i.e. input light intensity) is given in terms of photons per 6.5 µm pixel of each sensor type.

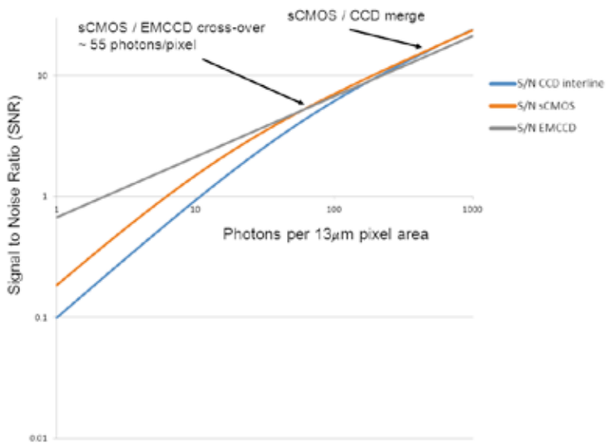
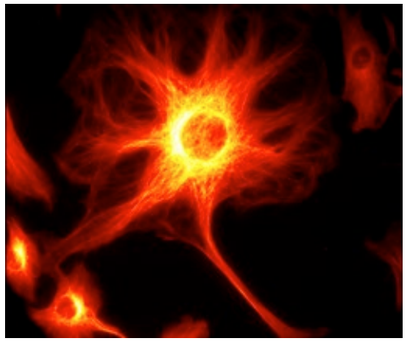


Figure 6 - Theoretical Signal to Noise plot comparisons for sCMOS vs Interline CCD vs back-illuminated EMCCD sensors. For purposes of a objective comparison, it is assumed that the ~6.5 µm pixels of the sCMOS and Interline CCD sensors are 2 x 2 binned in order to equal a 13 µm pixel of a back-illuminated EMCCD.

5.5 Megapixel sCMOS



1.4 Megapixel Interline CCD

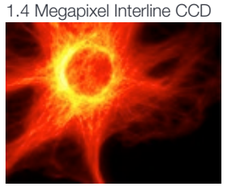


Figure 7 - Field of view comparison of two technologies; x60 magnification; 1.25 NA; 5.5 megapixel Andor sCMOS vs 1.4 megapixel Interline CCD (each have ~ 6.5 µm pixel pitch). sCMOS is capable of offering this larger field of view @ 100 frame/s with 1.3 e⁻ read noise.

5.5 Megapixel sCMOS

1.4 Megapixel Interline CCD

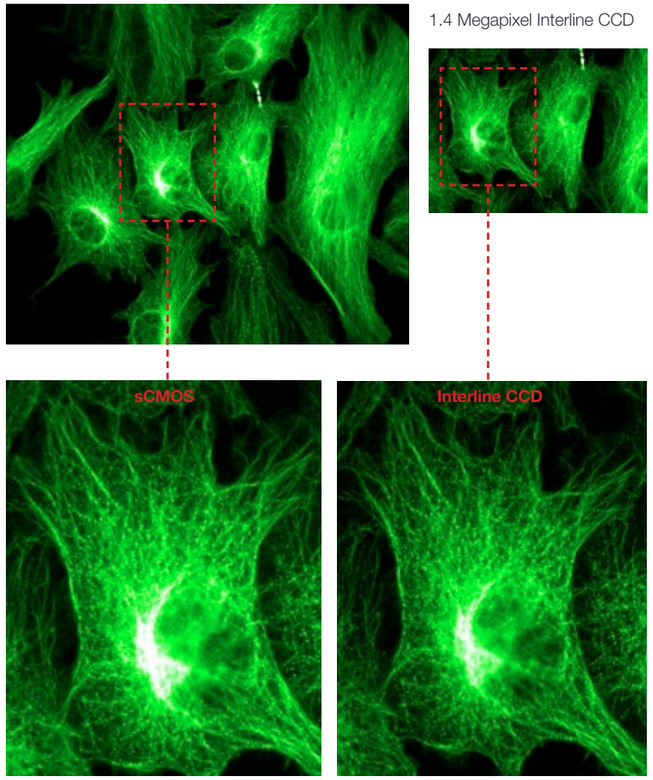


Figure 8 - Field of view and resolution comparison of two technologies; x100 magnification; 1.45 NA; 5.5 megapixel Andor sCMOS vs 1.4 megapixel Interline CCD (each have ~ 6.5 µm pixel pitch).

Notes...

Notes...

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Andor products are regularly used in critical applications and we can provide a variety of customer support services to maximise the return on your investment and ensure that your product continues to operate at its optimum performance.

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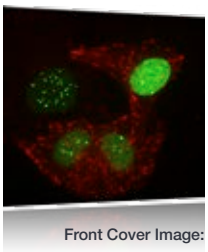

Front Cover Image:

Simultaneous dual colour imaging of live cultured cells with red labeled tubulin tips and green nuclear structures.

Captured with a Revolution XD running two iXon3 cameras on a TuCam camera adapter.

Some projects part financed by the European Regional Development Fund under the European Sustainable Competitiveness Programme for Northern Ireland.